TranscriptionPath™: A Method for RNA-free Transcription Profiling

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Active Motif's TranscriptionPath™ Assay is an alternate approach to RNA-based gene expression methods. The TranscriptionPath method utilizes Chromatin Immunoprecipitation (ChIP) to measure transcription rates as a function of RNA Polymerase II (RNAPII) occupancy across the genome. The assay can be combined with Next-Generation sequencing (TranscriptionPath-Seq) for a genome-wide readout, or it can be used with qPCR for a gene-specific readout. TranscriptionPath offers several advantages when compared to RNA-based approaches including the ability to look at early time points that are not accurately measured with mRNA approaches and the ability to measure gene expression in the same sample as was used to measure transcription factor (TF) binding by ChIP. In this study, TranscriptionPath data was compared to mRNA data to provide evidence that RNAPII occupancy is a reliable measure of transcription rates, and to show that TranscriptionPath data is rich in additional information such as locations of alternative transcription start sites (TSS) and identification of unannotated genes.

Introduction

Gene expression is a complex and tightly regulated process that is influenced by both transcription rates and mRNA degradation rates. For many genes the main regulatory mechanism of gene expression is at the level of transcription. This process can be described by a cycle of successive steps that includes: 1) the recruitment of an RNAPII containing complex; 2) initiation; 3) clearance of RNAPII from the site of initiation; 4) pausing; 5) escape from pause; 6) elongation, and 7) termination. Even though so many different events influence gene transcription, most approaches still measure only mRNA steady state levels, which gives no information regarding the mechanistic regulation of gene expression.

A more thorough and mechanistic understanding of gene regulation can be obtained by focusing on the actual process of transcription. This can be achieved by measuring RNAPII occupancy across the genome using chromatin immunoprecipitation. Using this approach, information can be collected on start site selection, pre-initiation complex (PIC) formation or pausing and transcription rates. Described below are the methodology and data interpretation, as well as the benefits of using this approach rather than RNA-based approaches.

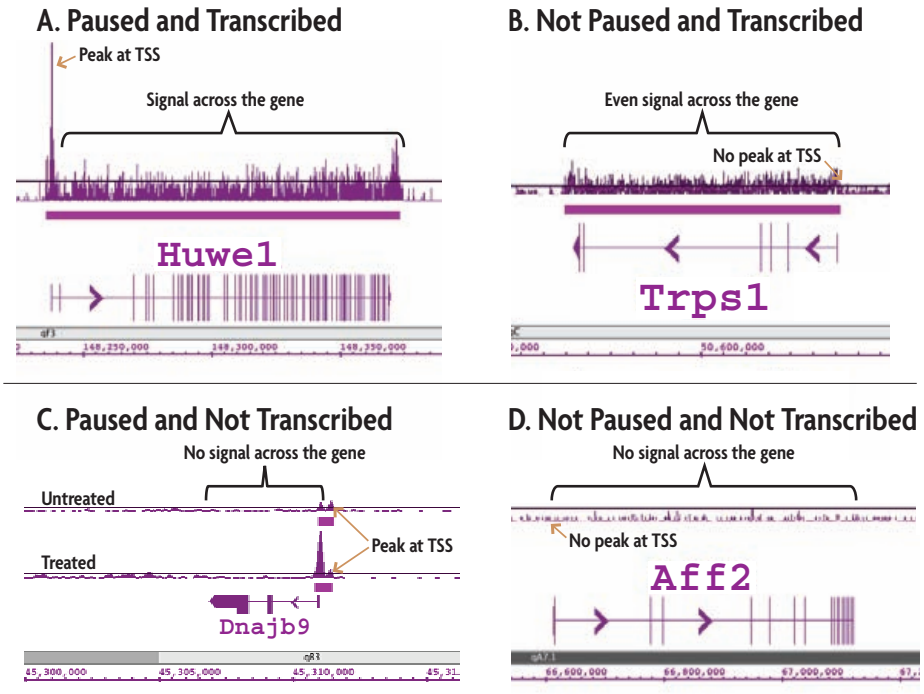

Figure 1: TranscriptionPath data reveals four states of RNAPII occupancy.
TranscriptionPath-Seq was performed using chromatin from mouse neuroblastoma cells. Four patterns of RNAPII occupancy were detected; examples are shown above. (A) Promoter Paused and Transcribed – Transcription is rate-limited post-initiation. (B) Not Paused and Transcribed – Transcription is rate-limited by RNAPII recruitment. (C) Promoter Paused and Not Transcribed – These genes are poised for activation, but are not transcribed. In this example, the poised pattern shown is inducible. (D) Not Paused and Not Transcribed – These genes are inactive.

Results

The Method and Data Readout

TranscriptionPath is based on the ChIP method utilizing an antibody that targets RNAPII. Four distinct patterns are observed when using ChIP-Seq to analyze the immunoprecipitated DNA (Figure 1, previous page). These are: A. Paused and Transcribed – This pattern is defined by a significant peak at the TSS followed by a lower, more even signal across the gene body. The peak at the TSS is indicative of either formation of the PIC or pausing following RNAPII release from the PIC. The elevated signal across the gene body is indicative of transcription of the gene. B. Not Paused and Transcribed – This pattern is defined by even signal across the gene body with no peak at the TSS. C. Paused and Not Transcribed – This pattern is defined by a peak at the TSS and no signal in the gene body. These genes have assembled the PIC and are poised for transcription, however the genes are not being transcribed. D. Not Paused and Not Transcribed – This pattern is defined by no recruitment of RNAPII to the gene body or TSS. In other words, these genes are inactive. The different patterns provide information on the mechanisms of transcription of each gene. Patterns A and C, which have RNAPII peaks at the TSS, have rate-limiting steps that occur after RNAPII recruitment. Pattern B, which does not display a RNAPII peak at the promoter, is rate-limited by the recruitment of RNAPII.

Some additional features of the TranscriptionPath signal that are worth noting concern RNAPII occupancy at the 3′ end of genes. RNAPII occupancy is strongly correlated with gene annotations, except at the 3′ end where signal often extends beyond where the 3′ end of the mature mRNA will be. This extended transcription is a known phenomenon and "extra" 3′ sequence will be cleaved during mRNA processing to yield the mature transcript[1]. Additionally, signals will often be elevated at the 3′ end of the transcribed region relative to the gene body. This pattern indicates slowed extension or polymerase pausing as transcription is terminated (Figure 2).

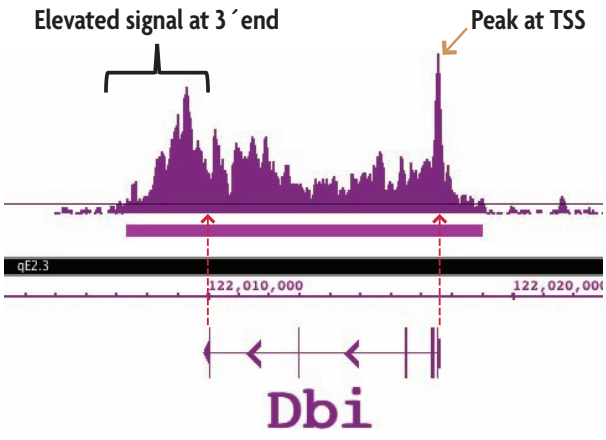

Figure 2: A representative TranscriptionPath gene profile.
TranscriptionPath-Seq was performed using chromatin from mouse neuroblastoma cells. Most genes will display an RNAPII peak at the promoter indicative of the paused state. RNAPII occupancy in the gene body tends to be fairly even across the gene body. Occupancy then increases toward the 3′ end of the gene and often extends beyond the 3′ gene boundary. The increased occupancy at the 3′ end is associated with pausing, as transcription is terminated. Red arrows mark the start and end of the annotation and serve to highlight these features in relation to the TranscriptionPath data.

Accessing Early Time Points

Many gene expression studies are initiated in an effort to understand how gene regulation is being altered in response to a stimulus such as a drug. Gene expression in these studies is often measured at time points beyond 24 hours, with six hours being considered an early measurement. However, gene expression changes at 24 hours are not primary events. The primary events occur within minutes of treatment, often with the induction of transcription factors that are transcribed, translated and imported back into the nucleus resulting in a second wave of transcriptional changes. In order to truly understand a cellular response it is important to understand the primary response, that is, the changes that are happening within minutes of treatment[2]. Measuring mRNA at these early time points has limitations, especially when studying transcripts derived from longer genes (*i.e.* genes over 100 Kb)[3]. Transcripts over 100 Kb will take over one hour to be fully transcribed, processed and exported to the cytoplasm where they can be detected. As a result, many changes in gene expression will be missed when using mRNA-based methods at early time points. The TranscriptionPath method can detect gene expression changes that are missed by mRNA detection methods because it measures RNAPII occupancy at DNA in real time. The occupancy of the RNAPII on the DNA is a surrogate readout for transcription rates and the TranscriptionPath data presented in this paper support this assertion. Additionally, studies by others support these findings and provide evidence that RNAPII occupancy is a reliable readout for gene transcription[4].

In order to demonstrate the ability of TranscriptionPath to assess gene expression at early time points and to compare it to mRNA-based methods, studies were initiated in a mouse pancreatic islet cell line treated with forskolin to stimulate changes in gene expression. TranscriptionPath DNA and mRNA from these cells were used to analyze specific genes of varying lengths. For TranscriptionPath, RNAPII ChIP was performed followed by qPCR using primers that were designed approximately 1500 bp 3′ of the TSS of each gene. Because the polymerase extends at a rate of approximately 1500 bp/min *in vivo*[5], this method enables us to detect changes in RNAPII occupancy within these genes at one minute after treatment. Figure 3 (next page) shows that for

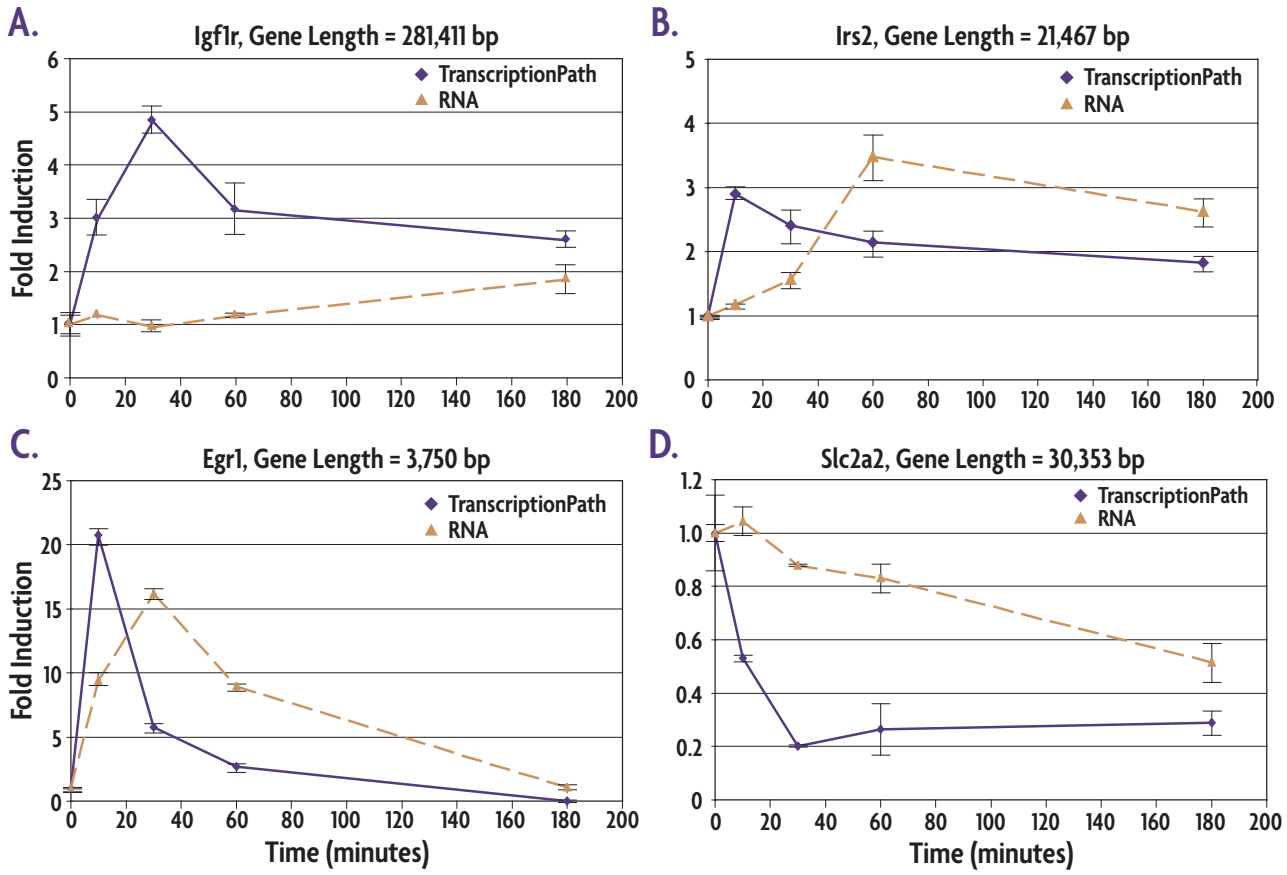

Figure 3: TranscriptionPath is better than mRNA-based methods at detecting changes in gene expression at early time points.
Mouse Min6 cells were treated with forskolin to induce changes in gene expression. Cells were fixed, chromatin was prepared and TranscriptionPath analysis was performed using qPCR primers specific to sequences approximately 1500 bp 3´ of the transcription start site (TSS). mRNA was measured by preparing cDNA and designing primers that span splice junctions. (A) Igf1r, (B) Irs2 and (C) Egr1 were all induced after treatment. For all of these genes the peak in transcription is detectable before the peak in mRNA, and the data shows that the longer a gene is, the farther the peak in mRNA lags behind the peak in transcription. Earlier detection and improved responsiveness is also observed with down-regulated genes. (D) TranscriptionPath analysis of Slc2a2 shows the greatest decrease in transcription at 30 minutes compared to three hours for RT-qPCR measurements.

three genes that were up regulated in response to treatment (Igf1r, Irs2 and Egr1), TranscriptionPath was able to detect changes that could not be detected using RT-qPCR at early time points. The most dramatic example was Igf1r, a gene that is 281,411 bp long. For this gene, TranscriptionPath was able to detect a three-fold increase in transcription at 10 minutes and a five-fold increase at 30 minutes. In contrast, RT-qPCR only detected a two-fold difference at three hours (Figure 3A). These results are as expected as RNAPII would require roughly three hours to fully transcribe a gene of this length. In contrast, as the Egr1 gene is only 3750 bp the full-length mRNA should be detectable within 2.5 minutes of treatment. Even so, the TranscriptionPath data shows earlier and more robust detection of gene expression for this shorter gene than RT-qPCR (Figure 3C).

For genes that are down regulated, the TranscriptionPath method can also detect changes earlier than mRNA-based approaches. This is illustrated by data on the Slc2a2 gene, which shows a 50% reduction in TranscriptionPath signal at 10 minutes and 80% reduction at 30 minutes (Figure 3D). It took three hours, or 18 times longer, to detect the same 50% reduction when measuring expression with RT-qPCR. The observed decrease in Slc2a2 mRNA over time is a reflection of the half-life of the mRNA since transcription of this gene, as shown by TranscriptionPath, was largely shut down. These data demonstrate the ability of the TranscriptionPath method to detect changes in gene expression that are not detectable with RNA methods at important early time points.

Combining TranscriptionPath with ChIP Reveals Functional Consequences of Transcription Factor Binding

ChIP is widely used to detect TF binding or histone modifications. When ChIP is combined with Next-Gen sequencing (ChIP-Seq), TF binding and modified histone occupancy can be mapped across the entire genome. However, mapping binding sites lack contextual information because the process of TF binding does not always lead to transcriptional activation or repression. In many cases binding results in a mixed response, with some genes being activated, some being repressed and some

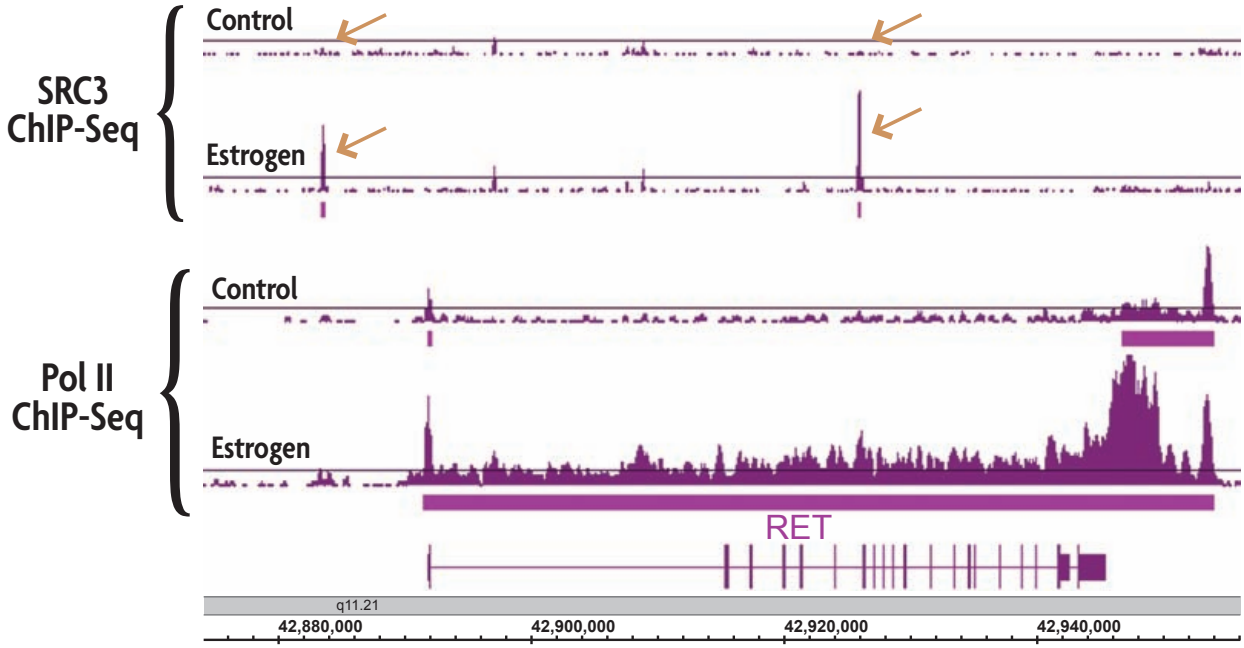

Figure 4: Induced TF binding correlates with TranscriptionPath-measured gene expression.
ChIP-Seq was performed using chromatin from control and estrogen-treated MCF-7 cells using antibodies directed against RNA Pol II and the estrogen-inducible transcription factor SRC3. Performing ChIP-Seq using an antibody against RNA Pol II produces a genome-wide profile of gene transcription rates and the TranscriptionPath ChIP-Seq data shows induced transcription of the RET gene after estrogen treatment (bottom 2 panels). Induced transcription of the RET gene correlates with the induced binding of SRC3 in the promoter and gene body of the RET gene (copper arrows in top 2 panels).

not changing their transcriptional response at all. These variable expression patterns are a reflection of the differences in the recruitment of other transcription factors and co-factors at each gene. In order to more fully understand the importance and/or function of individual TF binding events it is necessary to examine the effects of TF binding on transcription of each bound gene[6]. This type of analysis can be achieved by integrating ChIP data with gene expression data. The integration of TranscriptionPath data with ChIP-Seq data is advantageous in that A) the TranscriptionPath method can be performed on the identical chromatin sample that was used for TF ChIP, B) the TranscriptionPath data output is the same as the TF ChIP data output, thus simplifying the analysis, and C) the TranscriptionPath method is a direct readout of the effect of TF binding on transcription. This is in contrast to RNA measurements, which are influenced by other aspects, such mRNA half-life.

TranscriptionPath experiments were performed in parallel with ChIP-Seq to identify changes in SRC3 binding in response to estrogen treatment of MCF-7 cells. SRC3 binding events were identified that correlated with increased transcription of associated genes, decreased transcription and no change in transcription. Each of these three groups of genes were associated with different gene ontologies thus offering greater insight into the role SRC3 plays in estrogen responsiveness. A single example of SRC3 binding correlating with induced gene expression is presented in Figure 4, where 2 inducible SRC3 binding sites in the RET gene correlate with increased RET transcription.

Transcription Factors in Pathways

The cellular response to stimulation is a complex process that begins with the ability of the cell to sense changes in its extracellular environment and transmit the signal through the cell membrane to the cytoplasm. Intracellular signaling cascades then relay this information to the nucleus resulting in changes in chromatin structure and ultimately changes in gene regulation. The initial response of altered gene expression will include induced expression of transcription factors (for example, the well-characterized immediate early genes Fos, Jun, Myc and Egr1) that will ultimately be translated into proteins. These will be imported back into the nucleus and, through DNA binding, initiate a second wave of stimulated transcription[7]. These cycles of TF binding, which subsequently induce the expression of additional TFs, increase the complexity of temporal gene expression patterns. With this framework in mind, it is easy to see that gene expression profiles following treatment can be dramatically divergent when comparing one-hour treatments to 24-hour treatments. In order to illustrate the influence of timing and TF expression cycles on gene expression, the levels of transcription, TF binding and mRNA expression were each measured in a simplified model of a transcriptional network. In this example, MIN6 cells were treated with forskolin and the transcription of the immediate early

gene Egr1 was measured using TranscriptionPath. mRNA levels were determined using RT-qPCR. The data shows that Egr1 transcription peaks at 10 minutes (Figure 5A) while the peak in Egr1 mRNA occurs one time point later at 30 minutes (Figure 5B). The Egr1 message is translated into protein and imported to the nucleus where it binds DNA, resulting in the induction of a new set of genes. We identified Nab1 as one of the target genes of Egr1 using ChIP-Seq. Therefore Egr1 binding to the Nab1 promoter was studied further by performing Egr1 ChIP-qPCR on chromatin from all time points. Nab1 transcription levels were also determined using the TranscriptionPath-qPCR assay. The data shows that the timing of Egr1 binding to the Nab1 promoter occurs on the same time frame as Egr1 mRNA expression (Figures 5C and 5B) and that the timing of transcription of the Nab1 gene correlates nicely with Erg1 binding in the Nab1 promoter (Figures 5D and 5C). Lastly, Nab1 mRNA was measured by RT-qPCR and showed induction only at the three-hour time point (Figure 5E). Nab1 is a known transcriptional repressor[8] and the data show that its influence on transcription does not come into play until at least three hours after treatment. This detailed example of the timing of induced TF expression illustrates the importance of selecting appropriate time points for analysis and also demonstrates how induced TF expression continually influences gene expression.

Mathematical Modeling Shows TranscriptionPath is a Reliable Measure of Transcription

The data presented above shows that there is a clear correlation between TranscriptionPath data, TF binding and mRNA expression. To provide further evidence that TranscriptionPath accurately measures transcription rates, a mathematical model was developed that uses the measured TranscriptionPath data to predict mRNA levels in the cell. If the TranscriptionPath data is accurate, the predicted mRNA levels should overlap with the experimentally derived mRNA data points. This approach was used to model c-Fos mRNA levels using the TranscriptionPath experimentally determined transcription rates at 10, 30, 60 and 180 minutes after treatments along with published information on c-Fos half-life. A model assuming the c-Fos mRNA half-lives of 15 minutes[9] or 30 minutes[10] was used. If the half-life of c-Fos is set to 30 minutes (Figure 6A, next page), the predicted c-Fos mRNA levels nicely overlap the observed RT-qPCR data points. If the c-Fos half-life is set to

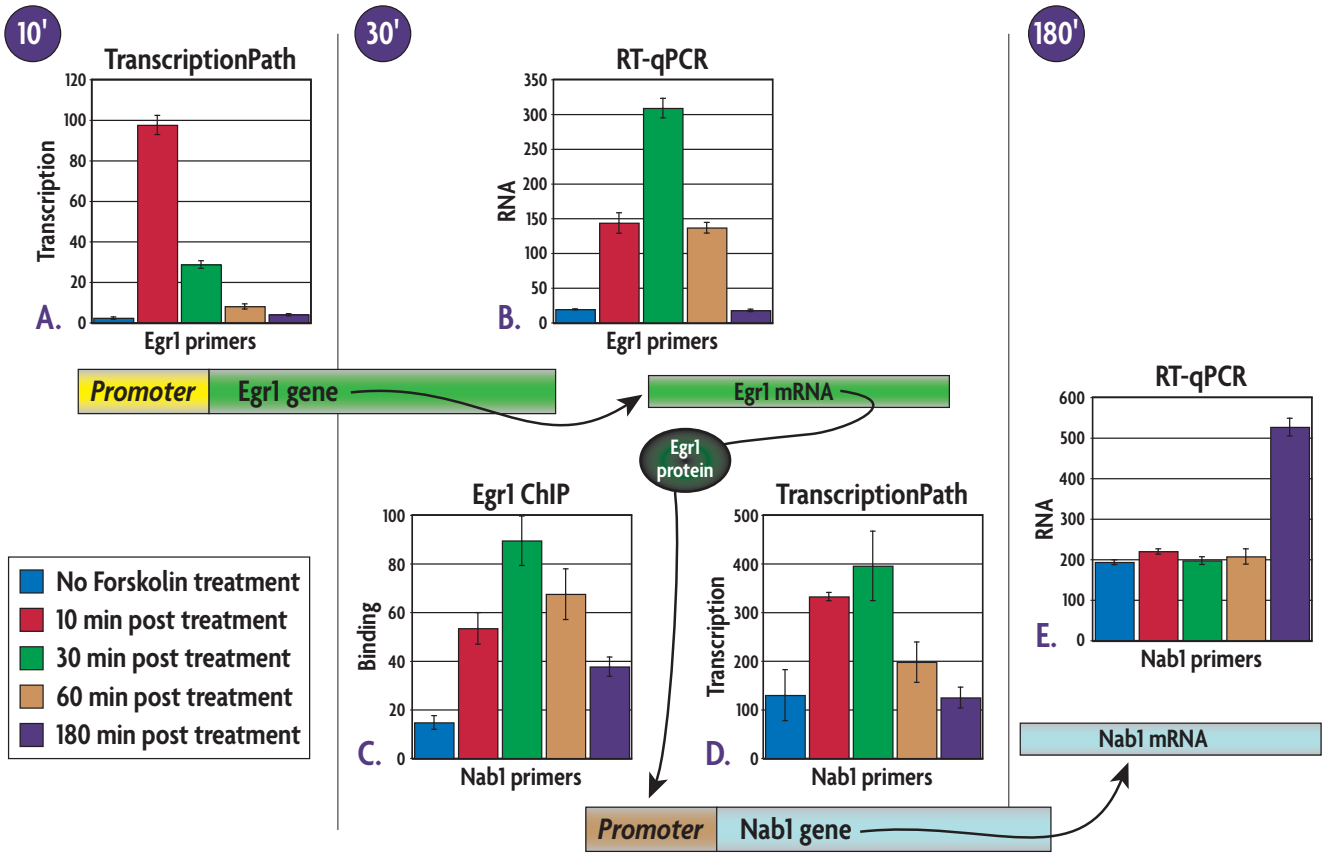

Figure 5: A detailed analysis of gene regulation using TranscriptionPath, TF-targeted ChIP and RT-qPCR.
Mouse MIN6 cells were treated with forskolin to induce gene expression changes. At 10, 30, 60 and 180 minutes after treatment either the cells were fixed and chromatin was prepared for ChIP, or total RNA was isolated for RT-qPCR. (A) TranscriptionPath was used to show a maximal increase in transcription of the Egr1 gene at 10 minutes. (B) When measured with RT-qPCR, the Egr1 mRNA peaked one time point later at 30 minutes. Egr1 ChIP-Seq identified Nab1 as a target gene for Egr1; (C) Egr1 ChIP-qPCR showed maximal induction of Erg1 binding to the Nab1 promoter at 30 minutes. (D) The timing of the transcription of the Nab1 gene mirrored the timing of Egr1 binding and also peaked at 30 minutes. (E) When measured with RT-qPCR, the induced expression of the Nab1 transcript was only detectable at three hours.

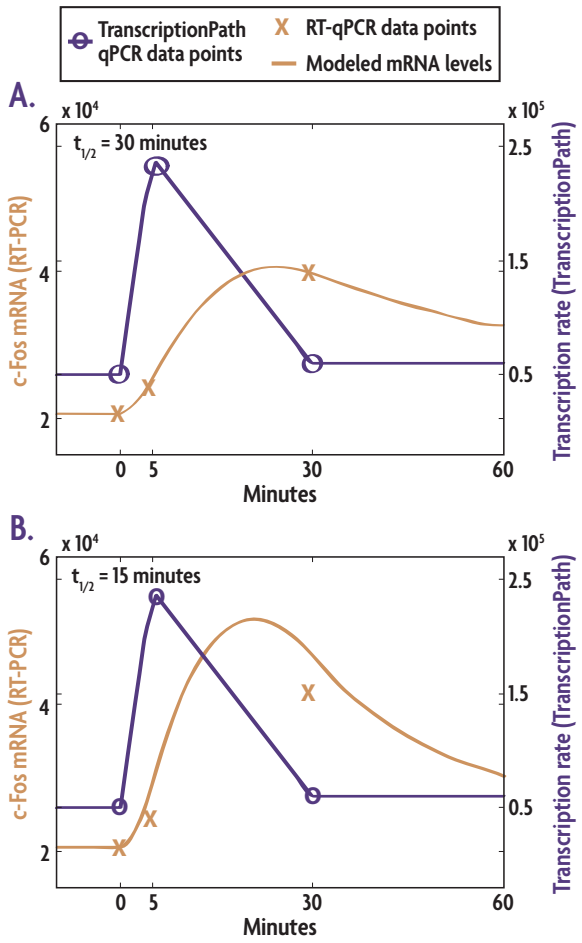

Figure 6: Gene expression models show that TranscriptionPath is an accurate measure of transcription rates.
Mathematical models of gene expression were developed at Thomas Jefferson University by Daniel Zak. (A) A model that assumes a c-Fos half-life of 30 minutes and using our TranscriptionPath data as input predicts mRNA levels that overlap precisely with our RT-qPCR data points. (B) A model that assumes a shorter c-Fos half-life of 15 minutes predicts mRNA levels that are shifted to the left of the actual RT-qPCR data points.

15 minutes (Figure 6B), the calculations are still valid and demonstrate peak mRNA expression occurring after the peak in the TranscriptionPath data, however the predicted data does not completely overlap the actual RT-qPCR data. These models support our assertion that TranscriptionPath is measuring actual transcription rates, as using the published c-Fos mRNA half-life of 30 minutes results in the predicted data precisely overlapping the experimental data.

Identification of Alternate Transcription Start Sites and Unannotated Genes

30-50% of all human genes use alternate TSSs[11]. The use of alternate TSSs not only contributes to genetic diversity and is needed for tissue-specific expression of different protein isoforms, but there is also evidence that aberrant use of TSSs of tumor suppressors and oncogenes is functionally linked to cancer[12]. mRNA detection using microarrays does not provide information of alternative TSSs because mRNA detection is typically based on hybridization to probes complimentary to the 3´ end of the message. Using the TranscriptionPath method for gene expression studies has the added advantage of providing information on TSSs, as Hao *et al.* demonstrated by detecting and confirming an alternate TSS for the Mef2c gene used in retinal rod photoreceptors[13]. The TranscriptionPath-Seq data show many examples of transcription initiation outside of the annotated TSS (Figure 7, next page). These alternate TSSs can occur within the existing annotation or far upstream of the annotated start site. Often the identified alternate TSS can be verified by searching additional gene databases. One further advantage that TranscriptionPath offers is the ability to detect transcription of unannotated genes. These two examples illustrate ways in which the TranscriptionPath data is rich with additional biological information beyond measuring transcription rates.

Discussion

Understanding any cell system, whether it is related to development, disease or response to treatment, requires an in-depth knowledge of gene regulation. Methods such as Nuclear Run-ons, Northern Blotting, PCR, Differential Display, SAGE, microarrays and RNA-Seq have evolved over the years to measure changes in gene expression. It is important to note that most of these methods measure steady-state RNA, which is influenced both by transcription rates and mRNA degradation rates. TranscriptionPath, on the other hand, measures the actual process of transcription. Therefore the information gained is more mechanistic, yielding a more direct readout of cellular response.

One advantage of looking at gene expression using TranscriptionPath is its increased sensitivity at short time points. The average human gene is 27 KB[14] and, assuming a transcription rate of 1500 bp/min, changes in mRNA expression for the average gene will not be detected until 18 minutes after treatment. Many genes are more than 100 Kb, thus requiring more than one hour until mRNA changes can be detected. The ability to look directly at transcriptional changes within minutes simplifies pathway analysis because it can be used to examine the immediate and direct effects of a treatment without the influence of secondary and tertiary effects that may occur when looking at later time points. In addition, the assay can be performed across multiple species since the carboxy terminal domain of the RNA polymerase is well conserved. To date, we have used TranscriptionPath in human, mouse, rat, *D. melanogaster*, *A. thaliana*, *C. elegans*, *Z. mays* and *S. cerevisiae*.

In addition, the assay may also be advantageous in situations where mRNA integrity is compromised or mRNA isolation is difficult or impossible. For example,

in FFPE samples the mRNA has typically been degraded, so RNA-based methods are not reliable. However, recent studies have shown that it is possible to isolate chromatin from FFPE samples and that the chromatin can be used in ChIP[15]. Therefore, TranscriptionPath could potentially be a consistent method by which gene expression studies could be performed using FFPE samples. A second application for TranscriptionPath, where mRNA analysis is not an option, is in conjunction with methods that allow for the isolation of cellular sub-populations from complex heterogeneous tissues. These protocols result in the isolation of nuclei, not whole cells, and TranscriptionPath would provide a means of gene expression profiling from these populations of nuclei[16].

TranscriptionPath was developed as an alternate approach to RNA-based methods of gene expression and provides a number of advantages that include the ability to 1) perform TF ChIP and get gene expression data using the exact same sample; 2) look at changes in gene expression at short time points after treatment; 3) directly measure transcription without the influence of mRNA half-life; and 4) detect alternately used TSSs and unannotated genes. In addition, this technology may prove to be useful in gene expression studies in FFPE samples and in gene expression studies where nuclear isolation is required.

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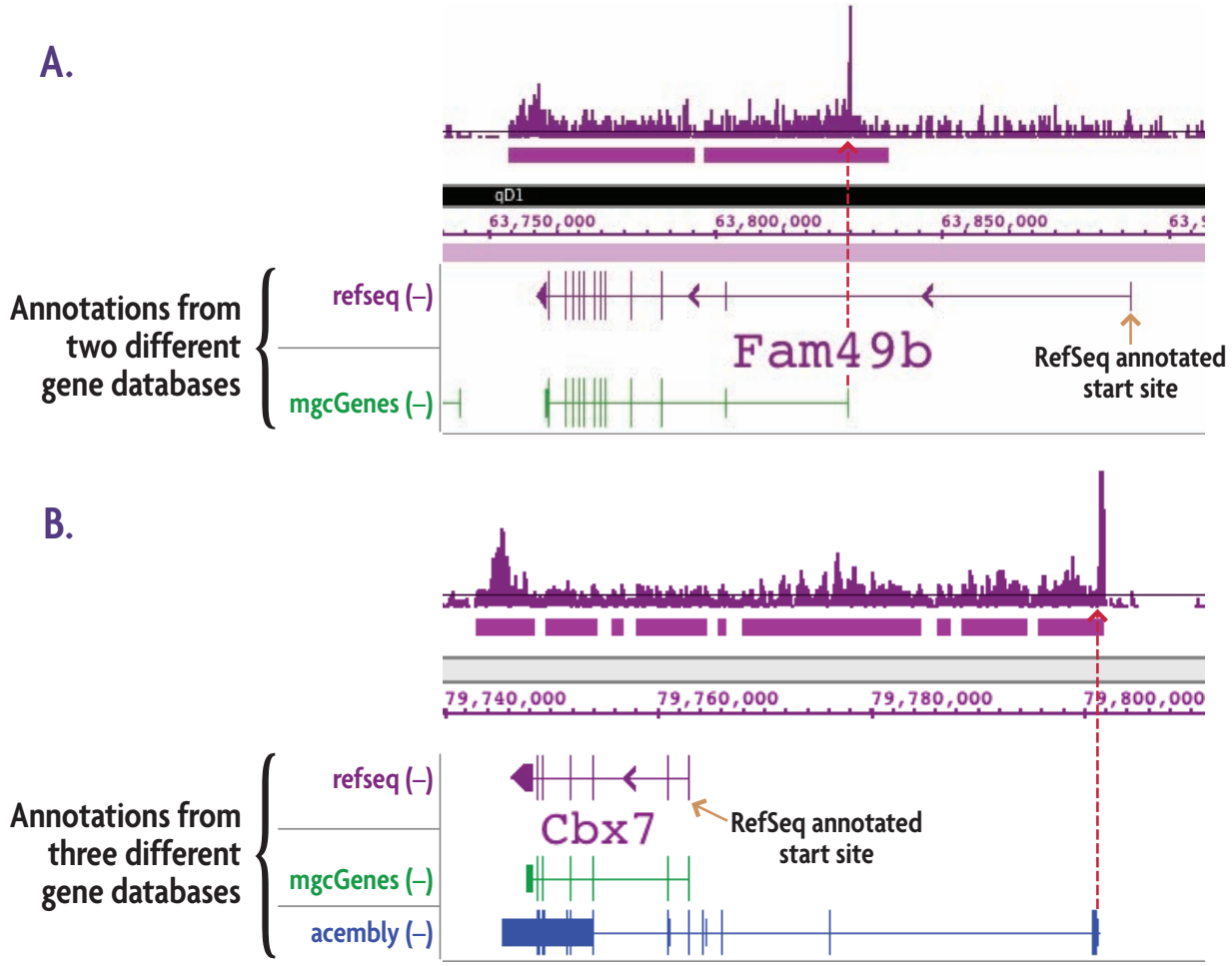

Figure 7: Alternate transcription start sites (TSSs) revealed by TranscriptionPath.
TranscriptionPath-Seq was performed using chromatin from a mouse neuroblastoma cell line. The data presented above is from two genes with transcription initiation in areas of the genome that do not correspond to their respective start sites in the RefSeq gene database (purple gene annotations). (A) Comparison to an alternate gene database (mgcGenes, green gene annotation) reveals a known alternative transcription start site for Fam49b that corresponds to the transcription start site detected with TranscriptionPath. (B) Comparison to the RefSeq and mgcGenes databases shows the same annotations for Cbx7, but comparison to a third gene annotation database (Acembly Genes, blue gene annotation) reveals a known alternative start site for Cbx7 that corresponds to the transcription start site detected with TranscriptionPath. The red arrows show that the start sites from the alternate gene databases correspond precisely with location of paused RNAPII.

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