The findings of the FANTOM3/Genome Network project have redefined the landscape of the mammalian transcriptome by introducing an extensive collection of novel cDNAs and millions of sequenced tags corresponding to 5′- and 3′-ends of mRNAs. This issue of PLoS Genetics includes a special collection of articles that explore the transcriptome complexity being revealed by work on the FANTOM3 dataset. Besides revealing staggering complexity, analysis of this collection is providing an increasing number of novel mRNA classes, expressed pseudogenes, and bona fide noncoding variants of protein-coding genes. In addition, new types of regulatory logic have emerged, including sense–antisense mechanisms of RNA regulation. This high-resolution cDNA collection and its analysis represent an important world resource for discovery, and demonstrate the value of large-scale transcriptome approaches towards understanding genome function.

The Era of Transcriptome Technology: From RNA to Function

After the completion of several genome sequences [1,2] the scientific community has been pondering what type of technologies are necessary for understanding the underlying biology of genomes. Two classes of novel technologies, one based on the hybridization of nucleic acids and the other on sequencing products from mRNA libraries, are already affecting the way we understand biological systems. Hybridization-based methods, such as genome tiling arrays [3–5], have some specific advantages: in a single experiment they can give a draft description of the transcriptome, or of genome elements selected by chromatin immunoprecipitation (ChIP) [6]. Although a general picture of the transcriptome can be produced quickly, important details such as transcriptional start sites (TSSs) cannot be accurately identified at single-base resolution, nor can such methods determine the exact exon connections and strand orientation, resulting in incomplete sequence information. Considering that 11% of short exons are not represented in Affymetrix tiling arrays, the overall rate of false negative and false positive exon detections is 20% and 5%, respectively (N. Maeda, S. Kondo, D. Sasaki, and Y. Hayashizaki, unpublished data). Despite these limitations, tiling arrays provide an important picture of the genome output, for example, that 41.5% of mRNA is restricted to nuclei, that there are 10-fold more transcribed sequences than there are annotated genes, and that 44% of the RNA sequences are never polyadenylated [3] and thus are missing from cDNA collections.

Methods based on full-length cDNA sequencing [7,8] are more intensive, but provide a picture at a higher resolution, including full sequence and exon–exon connectivity data [9–12]. Because large-scale full-length cDNA sequencing is expensive, novel mRNA tag technologies that are based on deriving sequence tags from full-length cDNAs have been developed (by the RIKEN group and others [13]). Three new technologies speed up the process of analysis: cap analysis of gene expression (CAGE) [14], gene signature cloning [11], and gene identification of signature [15]. The strategy of sequencing the 5′- and/or 3′-ends of transcripts enables increased throughput and brings transcriptome analysis to a new level. These technologies have been fully exploited in the FANTOM (Functional Annotation of the Mouse) and Genome Network (GN) Projects (see Box 1).

This special issue of PLoS Genetics is focused on transcriptome analysis done by GN and FANTOM3, carrying the torch from two recently published studies [11,12]. These studies force a paradigm shift in the understanding of the transcriptome. First, the studies find that 63% of the genome is transcribed from at least one strand (in contrast to the earlier belief that only 2% of the genome is transcribed into protein-coding mRNAs). Second, an unexpected amount of variation was found in alternative splice forms (65% of all transcriptional units [TU]s contain alternatively splicing variants), TSSs (which identify promoters), and polyadenylation sites. The number of TUs is somewhat reduced by the occurrence of gene fusion (exon sharing between neighboring genes), but the final number of TUs is still large (>43,000), because of novel mRNAs. Thus, the complex landscape of the

Citation: Hayashizaki Y, Carninci P (2006) Genome network and FANTOM3: Assessing the complexity of the transcriptome. PLoS Genet 2(4): e63. DOI: 10.1371/journal.pgen.0020063

Copyright: © 2006 Hayashizaki and Carninci. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: aa, amino acids; CAGE, cap analysis of gene expression; GN, Genome Network; S/AS, sense-antisense; TSS, transcriptional start site; TTS, transcriptional termination site; TU, transcriptional unit

Yoshhide Hayashizaki and Piero Carninci are in the Laboratory for Genome Exploration Research Group, RIKEN Genomic Sciences Center, RIKEN Yokohama Institute, Yokohama, Japan, and at the Genome Science Laboratory, Discovery and Research Institute, RIKEN Wako Institute, Wako, Japan.

* To whom correspondence should be addressed. E-mail: yoshihide@gsc.riken.go.jp
transcriptome is revealed, larger than ever before, and we are left with the daunting task of annotating its function and its usage in specific cells.

**Expanding the Bright Matter of the Transcriptome: More Proteins!**

Pulling together FANTOM3 and public datasets, no more than 2,200 completely novel proteins were identified, even when analyzing more than 158,000 cDNAs. More striking is the number of protein varieties discovered: combining all the splice variants gives at least 78,000 different mammalian proteins generated from approximately 20,000 protein-coding TUs [11]. Alignment of cDNA to the genome revealed splicing variations of three, six, or nine nucleotides [16] and identified novel splicing sites and splicing mechanisms. The work of van Nimwegen and colleagues improved the alignment of cDNA on the genome by developing the SPA algorithm, which allows a much finer alignment of cDNA to the genome. This advance allows us to better align the sequences, identify the 5'- and 3' -end mapping positions, and reduce the splicing boundary errors, thus improving the study of alternative splicing [17]. Based on the SPA algorithm, Chern et al. further analyzed the functionality of common short-length splice variations at the splicing site [18]. They identified small splice variations (mainly three nucleotides) that take place in 43.7% of all acceptor sites and 23.7% of all donor sites; these variations derive from stochastic binding of the spliceosome to the neighboring splicing site. It is unclear how these small-length splicing polymorphisms affect protein functions in various tissues, nor do we know the extent of this phenomenon in other organisms. We are used to thinking that genes produce compartment-specific proteins. But a new analysis by Davis et al. shows that alternative splicing causes the produced proteins to have different cellular compartment localizations in more than 8% of TUs [19]; determining in greater detail the cell-type specificity of such isoforms will require additional work. Further review of splicing in kinases and phosphatases found that 69% of these family members show alternative splicing [20], including variants that appear nonfunctional, but that with more careful analysis are revealed to be, respectively, decoy receptors and peptides resembling prolyl-tropic forms still capable of binding extracellular solutes. Although experimental validation is needed, such forms would be considered artifacts if we were not analyzing an extensive and redundant dataset.

An open question in the annotation of the transcriptome is the minimal length of a protein. Although this was previously arbitrarily set at 100 amino acids (aa), there is proof of the existence of shorter proteins. Applying the computer program CRITICA in a novel way, Frith and colleagues identified and experimentally verified a missing fraction of the proteome, which contains more than 3,000 candidate proteins (13% of the total number of proteins) that are shorter than 100 aa but longer than 50 aa, a limit below which CRITICA does not perform well, leaving the question unanswered as to whether there are even shorter unknown peptides [21]. Although many of these short proteins are potentially truncated protein variants, there are at least 1,240 that are truly novel short proteins. A part of all of these transcripts seems to be composed of genuine transcripts that seem to originate from within internal exons of longer transcripts, as described below (P. Carninci, unpublished data).

**Expanding the Dark Matter of the Transcriptome: Noncoding RNAs Require Our Attention**

In the past, the total number of genes was debated; early estimates ranged from 28,000 to 120,000 genes, based on expressed sequence tag clustering [22–24]. Today, such a large discrepancy can be at least partly explained by the discovery of the large number of noncoding genes and the variability of TU ends, which, before the genome and full-length cDNA sequences were made available, appeared as distinct entities. Additional knowledge of non-polyadenylated RNA based on tiling array technology can also contribute to the explanation [3]. The novel finding of 23,000 noncoding TUs and their prominent biological role in the regulation of gene expression has dramatically changed the traditional view of proteins as the only bioactive molecules, and emphasizes the need to modernize the central dogma.

To better distinguish between protein-coding and noncoding transcript, the FANTOM project has helped develop various computational tools to distinguish between non-protein-coding RNA and regular protein-coding mRNA. One of these tools is addressed in the current issue [25].

Two other papers further expand previous discoveries regarding the complexity of the transcriptome. Furuno et al. [26] analyzed cDNAs that may be constituted of fragments of larger RNAs that are unclonable because of their large size (such as the large noncoding transcripts Ar and Xis), and that are potentially cloned as 5'-3' truncated cDNA fragments through internal priming. Their search produced 2,700 large noncoding candidate transcripts, of which a small
function of expressed pseudogenes as remain open-minded about the suggest that we will need to continue to regulatory mechanisms. These data and are likely to participate in various otherwise characterized promoters, transcriptome—half of which are full-length cDNAs derived from expression and have identified 10,000 pseudogenes can be expressed and the discovery a few years back that this view might be incorrect was irrelevant [27–30]. A first indication transcribed or reintegrated into the testimony of old genes once considered pseudogenes to be a fossil only the tip of the iceberg. Recently identified and found to cover a large fraction of genes (72% of transcriptome. These pairs seem to co-expressed in the same cells and are co-expressed among co-expressed genes (nuclear versus cytoplasmic) and whether they correlation of cellular localization among co-expressed genes (nuclear versus cytoplasmic) and whether they are co-expressed in the same cells and from the same chromosome, in order to distinguish between a number of potential functional mechanisms. The observation that S/AS pairs are frequently clustered in complex genomic regions was made possible by using more than 158,000 cDNAs [11] (Figure 1). Engström et al. made a comprehensive catalog of complex loci, and have define the concept of “chain” for loci including at least three independent transcripts in S/AS relationship to each other or sharing a bidirectional promoter [37]. The rationale for such grouping is that such loci will be subject to common epigenetic relationships. They found that there are approximately 1,000 mouse–human conserved chains (7-fold more than known before) and that these include genes that are overrepresented in cancer, clearly revealing the need for further attention to expression dynamics in gene chains.

Biological Significance of Sense–Antisense

Antisense regulation of transcription is one of the many roles of RNA [11] and is one way a network of RNA molecules affects the entire organism’s phenotype. Short double-stranded RNA has been found to form regulatory chains at the genome, nucleoprotein, transcriptional, post-transcriptional, and translational level (for review see [34]). The extensive analysis of FANTOM3/GN showed that more than 36,000 sense–antisense (S/AS) pairs were encoded in the mouse transcriptome. These pairs seem to cover a large fraction of genes (72% of the total number of transcripts), including several important genes responsible for human genetic diseases and regulation of important cell functions such as cell cycle arrest and apoptosis [12]. Such prevalence of S/AS is confirmed with large-scale expression SAGE analysis, suggesting that more than 50% of the genes show S/AS transcription [35].

Interestingly, CAGE data showed that there is a preference for transcripts that map head-to-head in the genome, and in particular in the case of nuclear genes [12], suggesting a possible mechanism for transcriptional interference. In this issue, Seno and colleagues present an algorithm they developed to search for the expression of CAGE tags in the genome by looking for regional bias in regions that show significant co-regulation of transcription, including S/AS pairs [36]. By pooling together groups of CAGE libraries for similar groups of tissues (e.g., liver, lung, and macrophages, regardless of their condition), they identified S/AS transcription to be particularly overrepresented in certain loci, which strongly supports the hypothesis that transcriptional interference is a mechanism of transcriptional regulation. The next step regarding these data is to determine whether there is a correlation of cellular localization among co-expressed genes (nuclear versus cytoplasmic) and whether they are co-expressed in the same cells and from the same chromosome, in order to distinguish between a number of potential functional mechanisms. The observation that S/AS pairs are frequently clustered in complex genomic regions was made possible by using more than 158,000 cDNAs [11] (Figure 1). Engström et al. made a comprehensive catalog of complex loci, and have define the concept of “chain” for loci including at least three independent transcripts in S/AS relationship to each other or sharing a bidirectional promoter [37]. The rationale for such grouping is that such loci will be subject to common epigenetic relationships. They found that there are approximately 1,000 mouse–human conserved chains (7-fold more than known before) and that these include genes that are overrepresented in cancer, clearly revealing the need for further attention to expression dynamics in gene chains.

Variability within Transcripts

Highlights Complex Transcriptional Regulation Mechanisms

At a first glance, the FANTOM tags dataset has defined TSSs and transcriptional termination sites (TTSs) that exceed the number of TUs, with an average of five different TSSs and TTSs per TU (see Figure 2). Taken together, more than 181,000 different transcripts were identified for cDNAs, with proven evidence of TSSs and TTSs [12]. In particular, the collections of tags have helped in the identification of more than 290,000 TSSs in mouse. CAGE tags alone have identified 160,000 TSSs in mouse and 180,000 TSSs in human [38], expanding the number of well-annotated TSSs by several magnitudes [39]. This dataset has identified a functional dichotomy in mammalian promoters between CpG islands and TATA box promoters. Moreover, these tags have identified further “dark transcription matter” truly originating from 3' untranslated regions and coding exons. Analysis of expression clusters and their core promoter elements allows identification of regulatory elements and reconstruction of transcriptional networks and subnetworks after the activation of macrophages with lipopolysaccharides [40]. Bajic and colleagues analyzed mouse and human CAGE data and the local sequences around promoters, distinguishing them by their GC content at 5'- and 3'-ends of TSSs. Most of the promoters were indeed enriched in GC content, but there were also groups that were AT-rich in the same regions. The density of transcription factor binding sites and genes classified using Gene Ontology terms [41] highlights the basic differences that underlie transcriptional control of different gene categories, in agreement with our unpublished results. Expanded analysis of larger versions of such datasets will hopefully help not only to identify promoters, but also to predict their possible promoter activity, based on transcription factor binding site mapping and usage.

Taking advantage of CAGE's high resolution, Taylor and colleagues analyzed the evolution of core promoter elements [42]. Of particular interest is the fact that CAGE tags
reveal that human CpG-type promoters are under positive evolutionary selection, in contrast to TATA box promoters, which are evolving more slowly because of spacing constraints. As CpG promoters, TSSs are modular and driven by pyrimidine/purine dinucleotides [38], conferring greater transcription plasticity and thus accelerating evolution, because dramatic biological changes derive not only from protein mutation but also from differential expression levels. It would be interesting to apply CAGE technology to more evolutionarily distant vertebrates and invertebrates and further develop this concept of evolutionary rates as it relates to speciation.

**What Developments Lie Ahead?**

Tiling arrays and sequencing-based technologies have provided great insights—identifying several key roles of the RNA and transcription itself, and basic regulatory mechanisms. We have so far uncovered only a portion of the transcriptional complexity that exists, and when considering variation in tissues, cell development, and cell stages, we have barely scraped the surface of the unknown. For future studies, further technological development is needed, such as the recent application of tagging.
technologies to super-parallel sequencing analysis [43] as a part of the "$1,000 genome project." We need to be able to screen for the function of noncoding RNA, including the significance of cis and trans SIA/S interactions in living cells, and their roles in transcriptional interference, as epigenetic effectors, and yet unknown roles. Integration of these RNA functions and RNA variability into formally described gene models and their relation with phenotypes will then be required.

These datasets are complementary to and an integral part of the ENCODE project [44], a project devoted to the annotation of the entire human genome's regulatory elements. Integration of these two large-scale datasets will form the framework for future post-genomic studies. Hopefully, the future will see more comprehensive transcriptome projects flanking the basic genome sequencing projects, greatly enriching our knowledge and understanding of biology.

Acknowledgments

We would like to thank Nabiko Suzuki, Miki Nishikawa, Hiroko Himei, and Yukari Shigemoto for secretarial assistance, Ann Karlsson for scientific English editing, and
all of the GN and FANTOM consortium members for their contributions and efforts for the project. We would also like to thank Dr. A. Ward, Dr. M. Muramatsu, all members of the RIKEN Yokohama promotion office and the RIKEN office at the Wako main campus, the RIKEN executive committee, and the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) for their generous support.

We would like to dedicate this work to the memory of Dr. Verne Chapman, who

Funding. YH and PC are supported by a research grant for the RIKEN Genome Exploration Research Project from MEXT to YH, a grant of the Genome Network Project from MEXT, and the RIKEN strategic programs for R&D.

Competing interests. The authors have declared that no competing interests exist.

References

1. Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, et al. (2002) Initial sequencing and comparative analysis of the mouse genome. Nature 420: 520–562.
2. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, et al. (2001) Initial sequencing and analysis of the human genome. Nature 499: 860–921.
3. Cheng J, Kapranov P, Drenkow J, Dike S, Brubaker S, et al. (2005) Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. Science 308: 1149–1153.
4. Bertone P, Stolc V, Royce TE, Rozowsky JS, Lander ES, et al. (2004) Global identification of human transcribed sequences with genome tiling arrays. Science 306: 2242–2246.
5. Kapranov P, Cawley SE, Drenkow J, Bekiranov S, Strausberg RL, et al. (2002) Large-scale transcriptional activity in chromosomes 21 and 22. Science 296: 916–919.
6. Cawley S, Bekiranov S, Ng HH, Kapranov P, Sekinger EA, et al. (2004) Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell 116: 499–509.
7. Carninci P, Waki K, Shiraki T, Konno H, Shibata K, et al. (2005) Targeting a complex transcriptome: The construction of the mouse full-length cDNA encyclopedia. Genome Res 15: 1273–1289.
8. Carninci P, Shibata Y, Hayatsu N, Sugiyama Y, Hayashizaki Y, et al. (2006) The abundance of short sequence tags indicates 35,000 human genes. Nature 441: 634–639.
9. Liu J, Gough J, Rost B (2006) Distinguishing protein-coding from non-coding RNAs through support vector machines. PLoS Genet 2: DOI: 10.1371/journal.pgen.0020023
10. Engstrom PG, Suzuki H, Ninomiya N, Akalin A, Sessa I, et al. (2006) Complex loci in human and mouse genomes. PLoS Genet 2: DOI: 10.1371/journal.pgen.0020047
11. Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa S, et al. (2006) Genome-wide analysis of mammalian promoter architecture and evolution based upon human and mouse CAGE data. Nat Genet. In press.
12. Kasukawa T, Kai C, Kawai J, Carninci P, Hayashizaki Y, et al. (2005) A method for similarity search of genomic positional expression using CAGE. PLoS Genet 2: DOI: 10.1371/journal.pgen.0020023
13. Nakanishi M, et al. (2006) Antisense identification of promoter usage. Proc Natl Acad Sci U S A 103: 1559–1563.
14. Harbers M, Carninci P (2005) Tag-based approaches for transcriptome research and genome annotation. Nat Methods 2: 495–502.
15. Engstrom PG, et al. (2006) Transcriptional network dynamics in macrophage activation. Genomics. In press.
16. Bai J, Dike S, Brubaker S, Carninci P, Katayama S, et al. (2006) Comprehensive promoter analysis. Nucleic Acids Res 34: D632–D636.