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# Systematic analysis of transcribed loci in ENCODE regions using RACE sequencing reveals extensive transcription in the human genome

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Published: 3 January 2008

*Genome* ***Biology*** 2008, **9:**R3 (doi:10.1186/gb-2008-9-1-r3)

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2008/9/1/R3

Received: 7 November 2007
Revised: 6 December 2007
Accepted: 3 January 2008

## Abstract

**Background:** Recent studies of the mammalian transcriptome have revealed a large number of additional transcribed regions and extraordinary complexity in transcript diversity. However, there is still much uncertainty regarding precisely what portion of the genome is transcribed, the exact structures of these novel transcripts, and the levels of the transcripts produced.

**Results:** We have interrogated the transcribed loci in 420 selected ENCyclopedia Of DNA Elements (ENCODE) regions using rapid amplification of cDNA ends (RACE) sequencing. We analyzed annotated known gene regions, but primarily we focused on novel transcriptionally active regions (TARs), which were previously identified by high-density oligonucleotide tiling arrays and on random regions that were not believed to be transcribed. We found RACE sequencing to be very sensitive and were able to detect low levels of transcripts in specific cell types that were not detectable by microarrays. We also observed many instances of sense-antisense transcripts; further analysis suggests that many of the antisense transcripts (but not all) may be artifacts generated from the reverse transcription reaction. Our results show that the majority of the novel TARs analyzed (60%) are connected to other novel TARs or known exons. Of previously unannotated random regions, 17% were shown to produce overlapping transcripts. Furthermore, it is estimated that 9% of the novel transcripts encode proteins.

**Conclusion:** We conclude that RACE sequencing is an efficient, sensitive, and highly accurate method for characterization of the transcriptome of specific cell/tissue types. Using this method, it appears that much of the genome is represented in polyA+ RNA. Moreover, a fraction of the novel RNAs can encode protein and are likely to be functional.

## Background

Recent studies [1-5] have revealed that the composition and structure of the mammalian transcriptome is much more complex than was previously thought. Large-scale RT-PCR analysis to determine the structure of transcripts produced from exons of known human genes has shown that multiple transcripts are produced from most gene loci (an average of more than five was reported by Harrow and coworkers [6]). In many cases the 5' ends of these alternate transcripts are located more than 100 kilobases upstream from the previously known start site [1]. Likewise, systematic analysis of cloned mouse and human cDNAs revealed that many more transcripts than previously appreciated are transcribed from each known gene locus [7-9]. One source of complexity is alternative 5' ends; recent studies indicate that there are at least 36% more promoters than was previously recognized [10-14].

In addition to the diversity of transcripts from known loci, it appears that much more of the human genome is transcribed than was previously appreciated. Probing of tiling arrays with cDNA probes has indicated that there are at least twice as many transcribed regions of the human genome than had previously been annotated [3,15-18]. Rapid amplification of cDNA ends (RACE) analysis using primers designed to these novel transcribed regions (called transcriptionally active regions [TARs] or TransFrags) followed by hybridization to arrays confirms the transcription of these regions. However, this array analysis does not reveal information concerning transcript structure or abundance. The large number of these transcripts along with the fact that many long transcripts are produced suggest that much of the human genome is transcribed, at least at some level.

The different cDNA and tiling array studies to analyze transcription have also revealed extensive antisense transcription in mammalian genomes [2,19]. One concern is that these studies often use reverse transcription to create single-stranded cDNA, but this may also cause second strand synthesis. Thus, it is unclear whether the detected expression from the second strand is due to *bona fide* antisense transcription or a result of a probe made for the second strand.

These various studies have raised many more questions than have been answered. How much of the human genome produces transcripts that are present in the mRNA population? What is the nature of the transcripts produced by the novel transcribed regions? What fraction of novel transcribed regions is likely to be protein coding? What is the level of transcripts produced from the novel transcribed regions? Finally, how much antisense transcription occurs in human cells?

In an effort to address some of these questions and thereby better characterize the human genome and its gene annotation, we have systematically analyzed the transcribed loci in 420 selected portions of the ENCyclopedia Of DNA Elements (ENCODE) regions using 5'-RACE and 3'-RACE sequencing. The ENCODE regions are 44 regions that comprise 1% of the human genome and have been highly characterized with respect to transcripts and transcription factor binding [1]. Highly sensitive RACE sequencing provides new insight into the human genome and its transcription. We found that many genes not known to be expressed in a particular cell type produce properly spliced low abundance transcripts. We also found that in some cases the purported antisense transcription is likely to be an artifact of the reverse transcription reaction. Additionally, we systematically analyzed, for the first time, the structure and level of transcripts produced from many novel transcribed regions and from regions that were not known to be transcribed. RACE sequences derived from novel TARs showed that these regions are highly connected, and revealed the structure of several potential novel protein coding transcripts. Finally, we uncovered transcription in previous nontranscribed regions of the genome, demonstrating that much of the genome is transcribed. Overall, these studies significantly enhance our understanding of the transcriptome of the human genome.

## Results

### Overview of 5'-RACE and 5'-RACE sequencing experiments in selected ENCODE regions

We have studied the transcripts produced from annotated gene regions, novel TARs previously identified by high-density oligonucleotide tiling arrays, and regions that were not previously shown to be transcribed (nonTx regions) using 5'-RACE and 3'-RACE and DNA sequencing [15,18,20]. The chromosomal regions for our analysis are primarily from the ENCODE regions of chromosome 22, which is particularly well annotated, as well as additional ENCODE regions on chromosomes 11 and 21. The RNAs analyzed were from NB4 acute promyelocytic leukemia cells, HeLa cells, and placental tissue. Both polyA+ and total RNA were used. A summary of the experiments performed is presented in Table 1.

In total, 420 regions were analyzed; primers to each strand were designed and subjected to 5'-RACE and 3'-RACE reactions for a total of 1,680 reactions. Approximately 80% of the reactions generated products that were detected by gel electrophoresis (see Additional data file 1 for examples); 25% of these reactions yielded heterogeneous products (smears). The entire PCR reaction was subjected to DNA sequence analysis, and approximately 40% of the sequence reads mapped to the expected locations of the genome and were therefore deemed as products derived for the intended locus (see Materials and methods, below, for details regarding mapping of RACE sequences to the genome and the fitness score assignment). The average length of these sequence reads is 516 base pairs (bp). As expected, primers designed in known exons gave the highest proportion of valid RACE products. This is followed by the primers designed to the novel TARs. The

**Table 1**

**Summary of RACE sequencing using polyA+ and total RNA from human cell lines and tissue**

| Experiment | Number of exon primers | Number of novel TAR primers | Number of nonTx primers | Number of sequence reads | Number of detected transcripts on the genome |
|---|---|---|---|---|---|
| 1: NB4 total RNA | 34 | 39 | 0 | 291 | 154 |
| 2: Hela polyA RNA | 0 | 59 | 0 | 273 | 112 |
| 3: placenta total RNA | 32 | 20 | 44 | 195 | 85 |
| 4: placenta polyA RNA | 0 | 96 | 96 | 591 | 147 |

nonTx, region not previously shown to be transcribed; RACE, rapid amplification of cDNA ends; TAR, transcriptionally active region.

nonTx regions gave the fewest RACE products (Figure 1). Similar results were observed with both polyA+ and total RNAs, as well as from human cell lines or tissue.

### RACE sequencing is highly sensitive in detecting transcripts expressed at a low level

We first analyzed the RACE sequences from eight known gene loci. For six of these loci we analyzed RNA from cells in which the gene was known to be expressed. For two genes, 5'-RACE and 3'-RACE reactions were performed using primers designed to the forward and reverse strand of each exon. For an additional four genes we analyzed a subset (1 to 8) of exons in the gene. As shown in Figure 2, the sequences of the known loci mostly matched the known annotations. For example, analysis of the *DRG1* and *FBXO7* genes, which are known to be expressed in NB4 cells, revealed cDNA sequences that matched the expected transcripts described in Refseq. In addition to detecting known transcripts, we also found novel isoforms. Some of these isoforms contained new exons whereas others contained different combinations of the known exons. An example is shown in Figure 2b for *FBXO7*. A novel exon was found for one of the RACE products and a novel combination was observed for another product. For the six genes analyzed we found evidence for 16 novel isoforms.

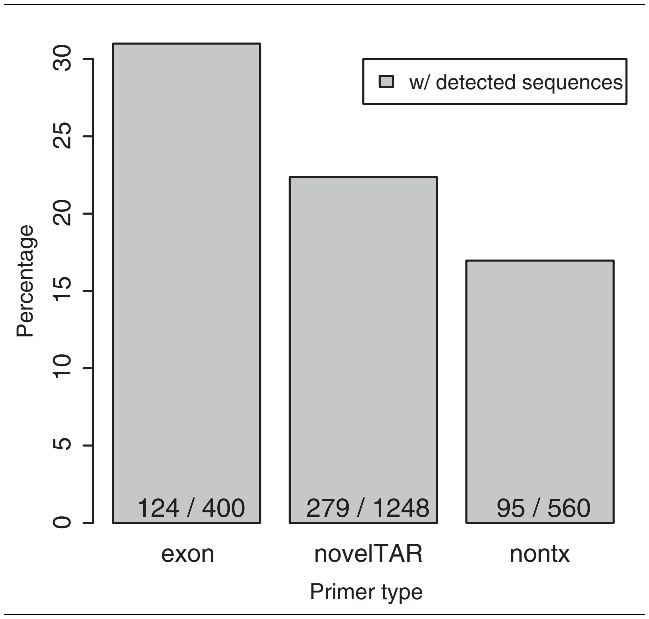


**Figure 1**
Frequency of PCR products obtained from different genomic regions. Primers designed to the sense and antisense strands of exons, novel transcriptionally active regions (TARs) and nontranscribed regions were used to generate rapid amplification of cDNA ends (RACE) products. The frequency of PCR products obtained is indicated. nontx, region not previously shown to be transcribed.

We also analyzed expression of two gene loci, namely *SYN3* and *TIMP3*, in cells in which their expression was not detected by tiling microarray analysis. *SYN3* and *TIMP3* are encoded on opposite strands from one another on chromosome 22. *SYN3* (*Homo sapiens* synapsin III mRNA) encodes a neuronal phosphoprotein that is involved in synaptogenesis and in the modulation of neurotransmitter release, and it is implicated in several neuropsychiatric diseases such as schizophrenia [21,22]. *TIMP3* encodes tissue inhibitor of metalloproteinase 3. Mutations in this gene have been associated with the autosomal dominant disorder Sorsby's fundus dystrophy [23]. NB4 RNA hybridization to high-density oligonucleotide tiling arrays did not produce signal above background in the *SYN3*/*TIMP3* region. With RACE sequencing a number of products were observed. Most RACE sequences (eight) matched that of the annotated RefSeq isoforms for *SYN3* (NM_003490.2). RACE sequences also revealed three other novel isoforms with exon skipping and intron inclusion (Figure 3a). Similar results were found for *TIMP3*. The presence of additional RNA isoforms suggests that additional messages are probably produced from each gene locus.

To gain a better understanding of why the *SYN3* and *TIMP3* genes were not detected by microarray analysis, we examined their expression level by real-time quantitative PCR. As shown in Figure 3b, the expression levels of *SYN3* and *TIMP3* are 1 × $10^4$ and 1 × $10^5$ times lower than that of the *HPRT1* transcript. *HPRT1* is expressed at low levels in various cell lines and tissue types, with fewer than 8 to 15 serial analysis of gene expression (SAGE) tags per 200,000 ($<10^{-5}$), according to the SAGE Anatomic Viewer [24]. Thus, the transcripts produced by Syn3 and TIMP3 in NB4 cells are present at an extremely low level.

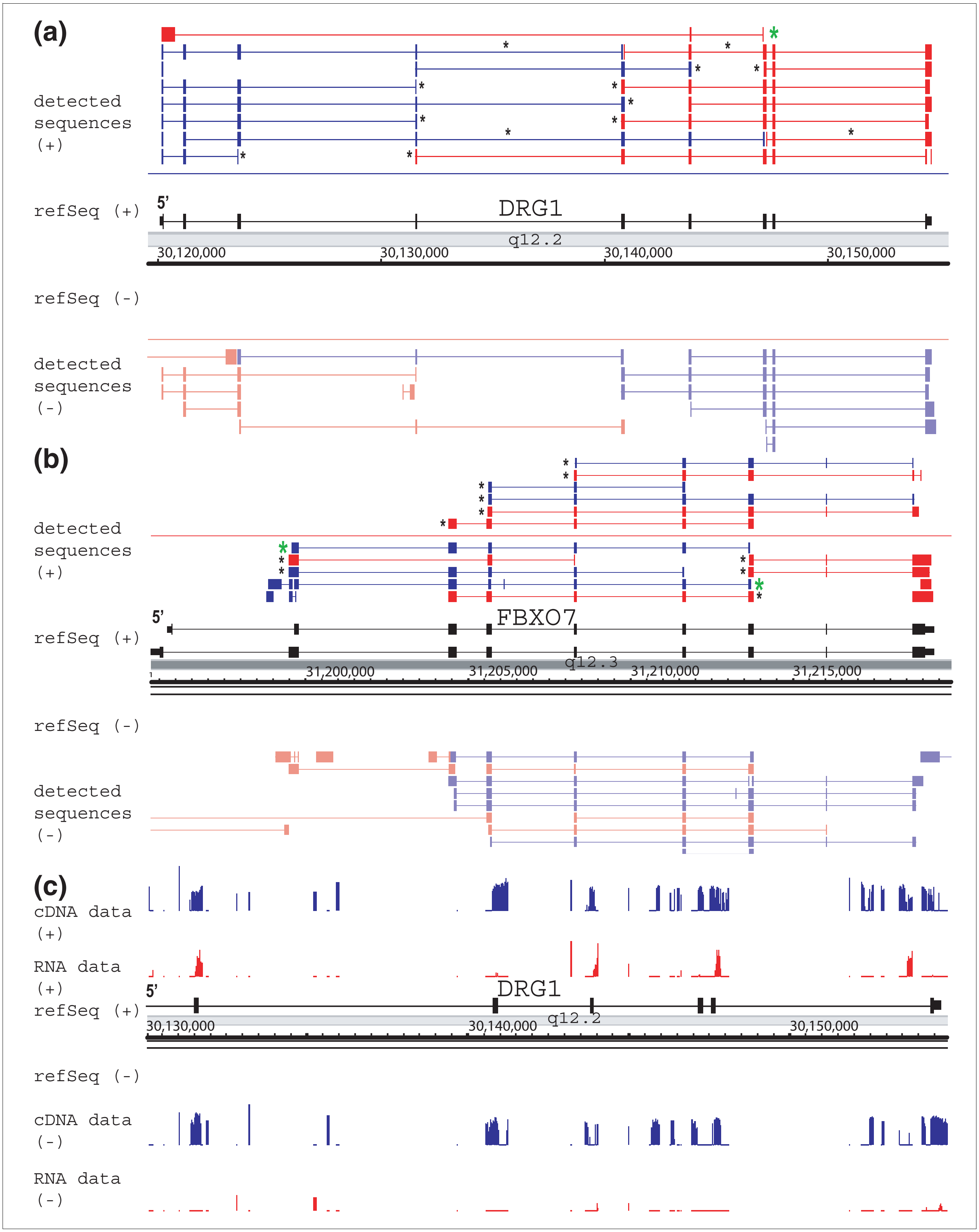


**Figure 2** *(see legend on next page)*

**Figure 2** *(see previous page)*
Distribution of RACE product sequences in the *DRG1* and *FBX07* regions. **(a)** *DRG1* Region and **(b)** *FBX07* region. Products from the sense strand (+) are shown in the top half of the panel. Products from the antisense strand are in the bottom half of the panel. Blue products are detected sequences from 5'-rapid amplification of cDNA ends (RACE); red products are detected sequences from 3'-RACE; black indicates refSeq; black asterisks indicate consensus splice sites (GT-AG, GC-AG, or AT-AC); and green asterisks indicate novel isoforms with more than 50% consensus splice sites. Note that the antisense products that lack consensus splice sites are indicated in lighter colors.**(c)** cDNA and RNA hybridization signals in DRG1 region. The blue tracks indicate the signals that were generated from hybridization of cDNA prepared from NB4 cells using reverse transcriptase to the strand-specific microarray. The red tracks indicate hybridization of RNA that has been labeled directly by chemical means, thus omitting the use of reverse transcriptase, to the strand-specific microarray. Products from the sense strand (+) are shown in the top half of the panel. Products from the antisense strand are in the bottom half of the panel.

The novel RNA isoforms from annotated genes were examined for their ability to produce novel protein isoforms. The 16 novel RNAs identified in this study can produce five novel protein isoforms.

### A number of antisense transcripts detected in multiple regions appear to be artifacts

Antisense transcription plays diverse and important biologic roles, and recent studies using reverse transcription based approaches have reported a large amount of antisense transcription in the human genome [16,19]. Our study employed primers to analyze transcription from both DNA strands and thus examined antisense transcription. In addition to detecting transcription from the expected DNA strand, the RACE experiments produced sequences from the complementary strand for five of eight known gene loci. These sequences were revealed in experiments using both natural tissue and cell lines. However, careful inspection revealed that in most cases (29 out of 35) the splice junctions of most of the antisense products are not consistent with the GT-AG, GC-AG, or AT-AC pattern. Instead, they merely mirror (reverse complement) the splice junctions of the sense products. Two examples are shown in Figure 2 for the *DRG1* region and the *FBX07* regions (known genes on the plus strand). In these regions large numbers of antisense products (14 and 21, respectively) were detected on the opposite strand from both 5'-RACE and 3'-RACE reactions. Most of the antisense products lack the (GT-AG, GC-AG, or AT-AC) consensus splice sequences. It therefore appears likely that many of these antisense products are derived from the *in vitro* reverse transcription reaction, where double strand cDNAs might have formed [25], or from a complementary RNA *in vivo* [16].

To investigate further whether the antisnese transcript may be an artifact due to reverse transcription, we employed a novel strategy, namely direct chemical labeling of RNA followed by strand-specific oligonucleotide tiling microarray analysis. As shown in Figure 2c for the *DRG1* locus, hybridization of cDNA prepared from NB4 cells using reverse transcriptase to the strand-specific microarray produced both sense and antisense signals. However, hybridization of RNA that has been labeled directly by chemical means, thus omitting the use of reverse transcriptase, usually yielded signals only from the annotated strand. This experiment indicates the much (but not all) antisense signal is directly tied to the use of reverse transcriptase and not likely to be present *in vivo*.

### Novel transcripts and their connectivity

In addition to examining annotated genome regions, we analyzed a large number of novel TARs by RACE sequencing in order to gain a better understanding of their structure, their connectivity to known genes, and whether they might encode proteins of significant length. In all, 856 RACE reactions were generated to 214 TARs of the ENCODE regions [18]. End sequencing of the 5'-RACE and 3'-RACE products on both strands of the genome revealed overlapping sense and antisense transcripts (Figure 4a). This is consistent with recent work by Kapronov and coworkers [5,11,26] using RACE microarray experiments, although they did not analyze the transcript structure. In addition to analyzing TARs, we designed primers to 140 regions not known to produce transcripts; 17% of the primers were able to generate a RACE product whose sequence mapped to the expected region (Figure 1). Control experiments lacking reverse transcriptase do not produce products, indicating that the products are derived from RNA and not contaminating DNA. This frequency of successful RACE products from nonTX regions is lower than in known exon or novel TAR regions, but it nonetheless indicates that a substantial fraction of the human genome produces RNA. The transcripts from the nonTx regions exhibited an interleaved distribution similar to those from the novel TARs (Figure 4b).

The majority (85%) of the RACE sequences from the TARs and nonTX regions map contiguously (without introns) to the genomic sequence. Products from primers that lie close together on the genome often overlap one another or known exons, suggesting extensive transcription throughout the entire region. In addition, whereas the RACE sequences derived from known exons are mostly connected with known exons, the sequences from nonTx regions are rarely connected to others (Figure 5a). Although some of the regions yield results consistent with discrete transcripts, many do not.

Approximately 16% and 11% of the products produced from TARs and nonTx regions, respectively, produce transcripts

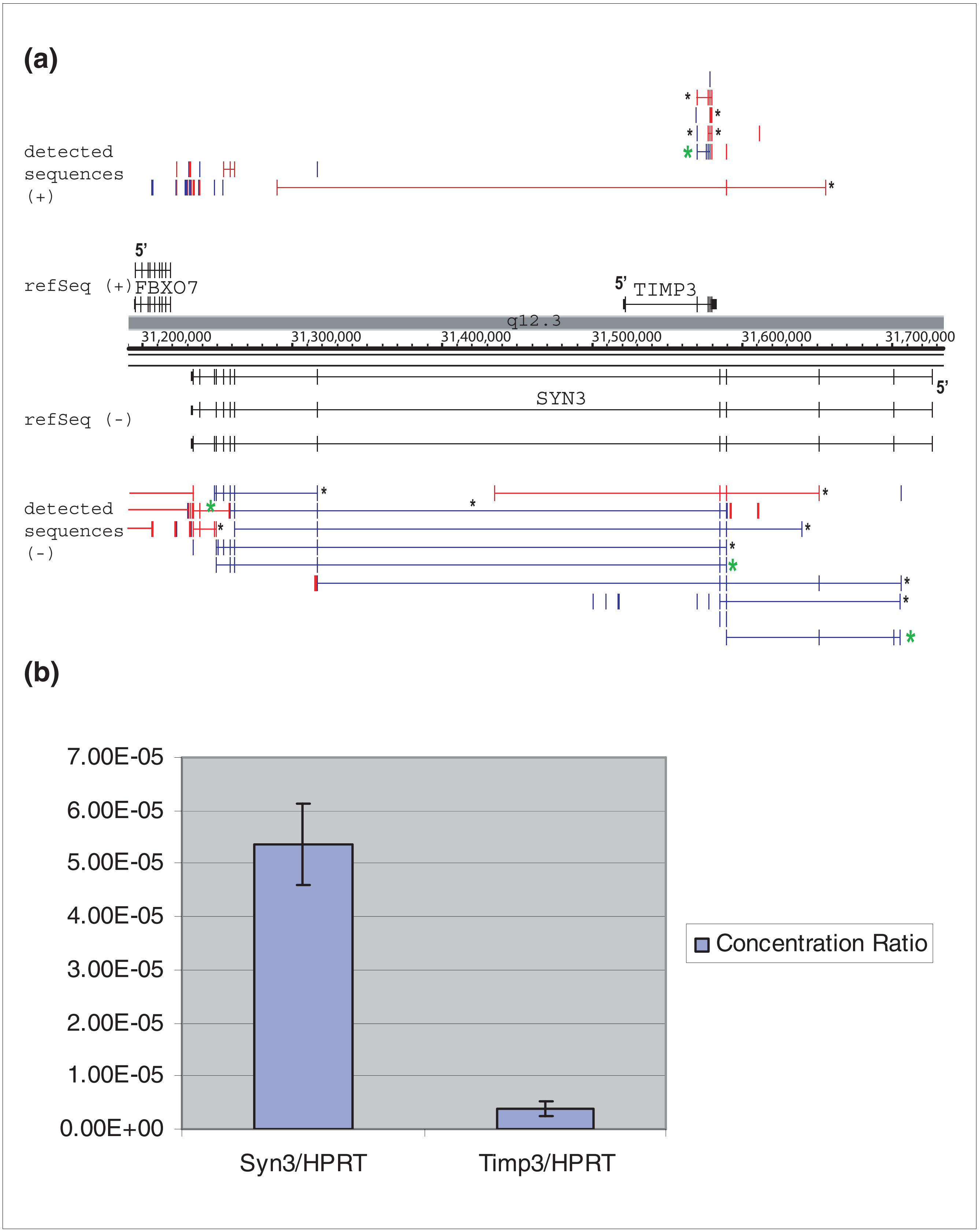


**Figure 3** *(see legend on next page)*

**Figure 3** *(see previous page)*
RACE sequencing can detect transcripts not previously detected by microarray analysis in NB4 cells. **(a)** Integrated Genome Browser (IGB) view *SYN3* and *TIMP3* rapid amplification of cDNA ends (RACE) products in NB4 RNA. **(b)** Real-time PCR quantification of *SYN3* and *TIMP3* transcripts relative to *HPRT1* in NB4 cells.

that are spliced with consensus GT-AG, GC-AG, or AT-AC splice sequences (see Consensus splice site analyses [under Materials and methods, below]; Figure 5b). This is in contrast to products produced from exons in which approximately 50% of the messages are spliced. Moreover, further analysis of the novel TARs revealed that the RNA sequences with consensus splice sites originated from regions with higher microarray signal intensity on average than the unspliced ones (Figure 5c). Microarray signal intensity of the nonTx regions is close to background for both spliced and unspliced RACE sequences.

### Several newly transcribed regions are likely to produce protein

In order to determine better whether the novel transcripts may be functional, we examined their ability to encode protein. The sequences of RACE products were analyzed with respect to whether they contain open reading frames (ORFs) and/or whether the potential protein coding sequences are homologous to those in the nonredundant protein database. For two spliced sequences and 25 unspliced sequences, potential ORFs were found that have at least 50 codons, and the predicted protein sequence was homologous to that of a known protein present in the nonredundant database with a BLASTX threshold score of 1 × $e^{-9}$ [27]. The 27 transcripts contain 20 unique proteins, and nine out of 20 protein encoding ORFs have a translational start and stop codon (11 of the 27 transcripts).

One example of a potential protein coding transcript is shown in Figure 6. The novel transcript 5NGSP2F8 detected by RACE end sequencing was properly spliced with a consensus pattern. It encodes a potential ORF that is 142 codons in length. Evidence for the transcript is also supported by a spliced expressed sequence tag (EST), although for 5NGSP2F8 the EST sequence contains a shorter ORF, presumably through DNA sequencing errors.

We examined the expression level of novel transcript 5NGSP2F8 using real-time quantitative PCR. The 5NGSP2F8 expression level is more than 1,000-fold lower than that of the *HPRT1* transcript, indicating that the gene is expressed at a low level (Figure 6b).

## Discussion

Even though it is estimated that only 20,000 to 25,000 protein coding genes exist in the human genome, the transcriptome is quite complex and contains protein coding, nonprotein coding, alternatively spliced, and antisense genes [28]. RACE sequencing has provided a sensitive means for probing the human transcriptome. We found that transcripts from known gene regions often matched the known gene annotation but that many additional novel transcripts were also detected. We were also able to detect both novel and known RNA transcripts from known genes that were not previously detected in NB4 cells using genomic tiling arrays. It is thus likely that many (and possibly the majority) of known genes are expressed and spliced in human tissues and cell lines, and that multiple transcripts are produced from most gene loci, at least at a low level.

In addition to many annotated exons, high-density oligonucleotide tiling arrays has identified a large number (8,958) of novel TARs located in both intronic regions and intergenic regions distal from previously annotated genes [15,18]. In this report, end sequencing of the 5'-RACE and 3'-RACE PCR products from novel TARs identified extensively overlapping and interconnected novel transcripts. Most of the RACE sequences from the novel TARs and the nonTX regions are unspliced. This is consistent with mouse transcriptome studies, which found the most obvious difference between coding and noncoding transcripts to be that a higher percentage (71%) of the noncoding transcripts are unspliced/single exons, as compared with protein coding transcripts (18%) [29]. Many human RACE products do not contain long ORFs, and thus the function of these transcripts is not known. They probably either represent nonprotein coding RNAs that may have structural, enzymatic, or regulatory functions; pre-mRNAs; or RNAs from genomic regions that are transcribed and present in polyA+ RNA but lack a function.

Although many of the novel RNAs do not have long ORFs, a subset of them do (about 9%). From our limited study we found 27 protein coding sequences that are not present in RefSeq but are likely to encode proteins based on the presence of a more than 50-codon ORF that is homologous to other proteins in GenBank. A small fraction (two out of 27) of these is spliced. Additional studies of the entire human genome are thus likely to expand the number of protein coding genes accordingly.

Complementary natural antisense transcripts exert control at many steps of gene expression in prokaryotes and higher eukaryotes from transcription to translation, including transcript initiation, elongation, mRNA processing, location, and stability [30,31]. Natural antisense transcripts may be involved in diverse biologic functions, such as development, adaptive response, viral infection, and genomic imprinting [32,33]. In recent years, a large amount of sense-antisense

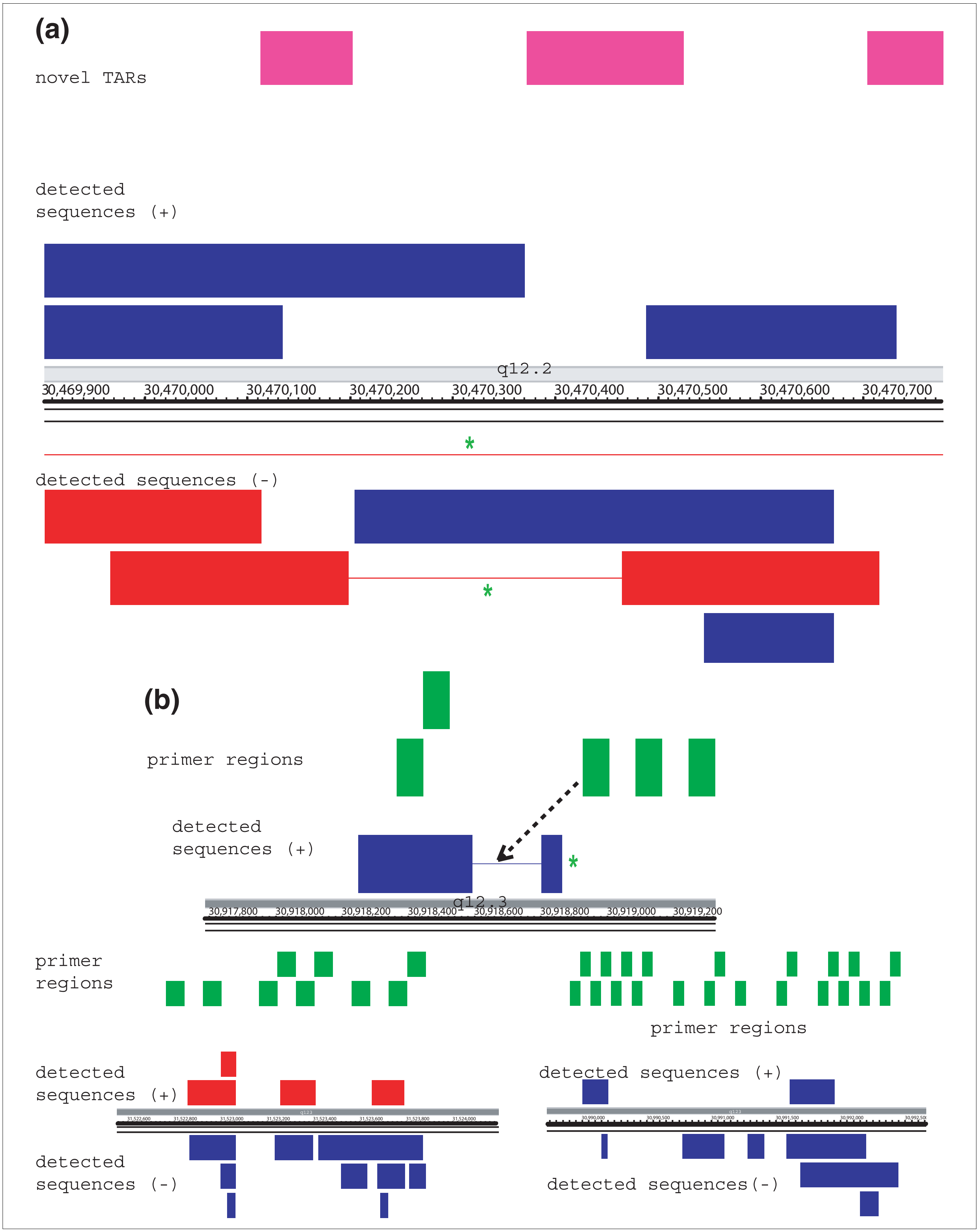


**Figure 4** *(see legend on next page)*

**Figure 4** *(see previous page)*
RACE products from novel TARs and nonTx regions. **(a)** novel transcriptionally active regions (TARs) and **(b)** regions not previously shown to be transcribed (nonTx regions). Pink indicates novel TARs, and green nonTx regions that the primers were designed from. Note that the products are primarily unspliced.

transcription phenomena have been reported in both human and mouse. In a mouse transcriptome study using the reverse transcribed cDNA libraries [19], it was indicated that as many as 72% of all transcriptional units have an antisense transcript. In humans, 61% of all transcribed regions were suggested to possess antisense transcript [16]. Our findings that some antisense transcripts lack consensus splice junctions and can be detected on strand-specific microarrays only in cDNA, but not directly labeled RNA, raises the possibility that many antisense signals are artifacts resulting from reverse transcription. The conditions that we used are similar to those used by most other laboratories, suggesting that low level second strand synthesis is likely to be present in many studies. Consistent with this, while our manuscript was under review, Perocchi and coworkers recently reported the presence of *in vitro* antisense synthesis in their cDNA preparations [25]. These findings indicate that much antisense transcription is due to *in vitro* synthesis and not *in vivo* cDNA synthesis, and therefore caution should be used in interpreting antisense messages. The fact that some antisense regions still hybridize to directly labeled RNA probes indicates that some antisense transcripts do exist *in vivo*.

RACE sequencing was able to uncover novel transcripts from nontranscribed regions where microarray experiments did not detect any transcription, indicating the RACE sequence is more sensitive. This is probably due to the fact that micorarray signals are dampened by cross-hybridization to short oligonucleotides on the array. This problem is especially acute for genes that have homologous pseudogenes and paralogs. RACE sequencing offers several other advantages relative to microarrays. Microarrays do not provide information about transcript structure, splicing patterns, or the ability of these regions to encode proteins. Only sequencing full-length cDNA can resolve these issues. The recent developments of massively parallel sequencing technology has the potential to expedite this process greatly [34-37]. A large number of sequences (400,000 250-bp reads for 454 sequencer [Roche Applied Science, Indianapolis, IN, USA] and >300 million approximately 30-bp reads for Solexa sequencer [Illumina Inc., San Diego, CA, USA]) can readily be obtained in a single run. Although still relative short, these reads have the potential to identify novel transcribed regions of the human genome, and the longer reads may help to identify new spliced variants [38].

As noted above, quantitative measurements of transcript expression reveals that two known genes (*SYN3* and *TIMP3*) are expressed at low levels even in tissues where they have no obvious role and cannot be detected by standard methods. Likewise, analysis of novel TARs and even random regions of the genome indicates that much of the genome produces transcripts that are present in polyA+ RNA, at least at a low level. Expression of these RNAs was $10^3$ to $10^5$ times lower than that of the *HPRT* gene. Assuming that *HPRT* is present at $10^{-5}$ (1 copy per 100,000 molecules of the total RNA) in total RNA, the novel transcripts we detected are present at $10^{-8}$ to $10^{-10}$ of the total RNA. The finding that much of the genome is likely to be expressed has previously been reported for yeast, for which evidence also exists that the RNA is translated [39,40]. As suggested previously, we speculate that the ability to express novel regions of the genome continuously could ultimately be useful in evolution for selecting new functions.

Our study highlights the enormous complexity of the human transcriptome and the vast amount of RNA transcripts generated both from alternative splicing and protein coding and nonprotein coding RNAs. The ability of RNA to encode protein and to serve a structural and regulatory role makes it a diverse molecule for mediating many functions. The remarkable complexity of RNAs of the human transcriptome coupled with their diverse functions may therefore help explain the dramatic increase of complexity in higher eukaryotes and phenotypic variation [41,42].

## Materials and methods

### Target selection

The regions of our analysis are selected mainly from the chromosome 22 ENCODE region, with additional targets in chromosome 11 and 21 ENCODE regions. Except for a few regions for test purposes, we selected most of the exon and novel TAR primer regions from among those expressed (cell type specific) regions in known exons and novel TAR regions detected by transcriptional tiling array experiments. The nontranscribed primer regions are selected in a tiled manner from among those regions that are neither known exons nor novel TARs.

### Primer design

We designed four primers for each targeted region, which can be exons of known gene, TAR, or previously identified untranscribed regions. Two gene-specific primers (GSP1 and GSP2) and two nested GSPs (NGSP1 and NGSP2) on both plus and minus strand were selected for each targeted region using a modified Primer3 program. The primers are 23 to 28 nucleotides long, with GC content of 50% to 70% and with Tm (melting temperature) above 70°C (optimally 73°C to 74°C). Self-complementary primers that could form hairpin were

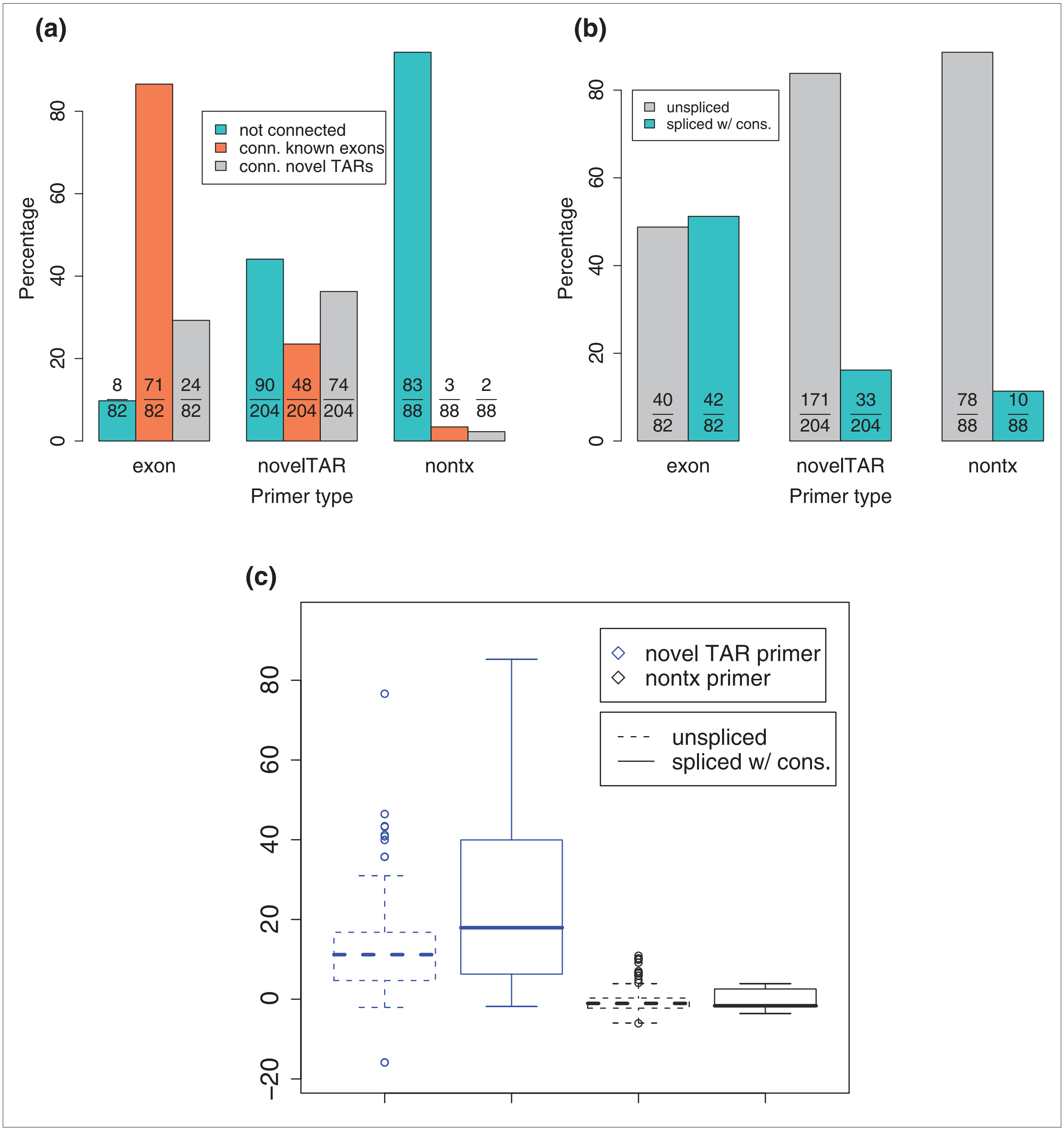
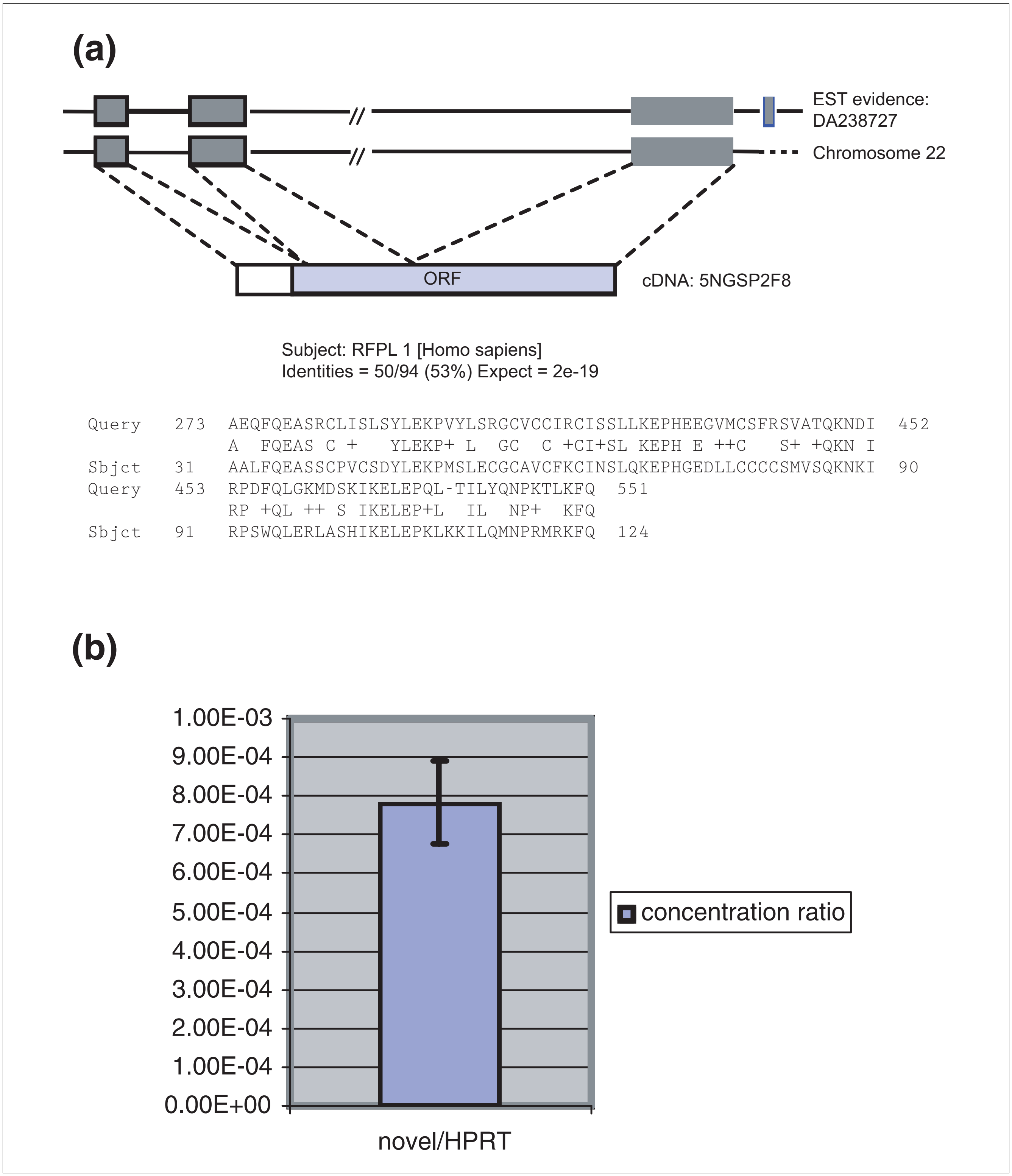


**Figure 5**

Features of the RACE products. **(a)** Connectivity of detected transcripts to known exons/novel transcriptionally active regions (TARs). **(b)** Frequency of splice and unspliced rapid amplification of cDNA ends (RACE) products derived from known exons, novel TARs, and untranscribed regions. **(c)** Average microarray intensities of regions encoding spliced and unspliced RACE products. nontx, region not previously shown to be transcribed.

avoided. We also voided complementarity between GSPs and UPM (universal primer A in the SMART RACE™ kit [Clontech, Mountain View, CA, USA]), particularly in their 3' Ends (UPM long: 5'-CTAATACGACTCACTATAGGGCAAGCAGT-GGTATCAACGCAGAGT-3'; UPM short: 5'-CTAATACGACT-CACTATAGGGC-3'). Complementarity between NGSPs and NUP (nested universal primer A), particularly in their 3' ends, was avoided (NUP: 5'-AAGCAGTGGTATCAACGCAGAGT-3'). The primers were mapped against the genome to ensure



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Query   273   AEQFQEASRCLISLSYLEKPVYLSRGCVCCIRCISSLLKEPHEEGVMCSFRSVATQKNDI  452
              A  FQEAS C +   YLEKP+ L  GC  C +CI+SL KEPH E ++C   S+ +QKN I
Sbjct   31    AALFQEASSCPVCSDYLEKPMSLECGCAVCFKCINSLQKEPHGEDLLCCCCSMVSQKNKI  90
Query   453   RPDFQLGKMDSKIKELEPQL-TILYQNPKTLKFQ  551
              RP +QL ++ S IKELEP+L  IL  NP+  KFQ
Sbjct   91    RPSWQLERLASHIKELEPKLKKILQMNPRMRKFQ  124
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**Figure 6**

Example of a novel transcript detected by RACE sequencing. **(a)** Novel transcript 5NGSP2F8 (with consensus splice site) has a potential open reading frame of 142 amino acids; also, there is spliced expressed sequence tag (EST) evidence for it. **(b)** Real-time PCR relative quantification of the novel transcript to *HPRT1* in placenta polyA+ RNA. RACE, rapid amplification of cDNA ends.

that they mapped to only one location (with identity <80% to other locations).

## 5'-RACE and 3'-RACE experiments, and end sequencing

Human NB4 cell line total RNA, Hela S3 polyA+ RNA, placenta total RNA, and polyA+ RNA (Ambion, Austin, TX, USA) were used in cDNA amplification by SMART RACE™ kit (Clontech), in accordance with the manufacturer's instructions [43]. 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA were synthesized using PowerScript (Clontech, CA, USA) or Superscript II (Invitrogen, CA, Carlsbad, USA) moloney murine leukemia virus reverse transcriptase and SMARTII A oligo (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3'), 5'-CDS primer A ([5'-(T)25V N-3' (N = A, C, G, or T; V = A, G, or C)]), or 3'-CDS primer A (5'-AAGCAGTGGTATCAACGCAGAGTAC[T]30V N-3' [N = A, C, G, or T; V = A, G, or C]). A total of 1 μg RNA was used in a final volume of 10 μl reverse transcription reaction (100 ng/ul). A reverse transcription reaction without reverse transcriptase was used as negative control to distinguish genomic DNA contamination. RACE was followed by PCR amplification using UPM and GSP1 or GSP2 on both strands of the genome. A 0.5 μl reverse transcription reaction from the above was used in 50 μl of PCR reaction in the Advantage™ 2 PCR Enzyme System (Clontech). Nested PCRs were performed using NUP (5'-AAGCAGTGGTATCAACGCAGAGT-3') and NGSP1 or NGSP2. One microliter of RACE PCR product was used in a 50 μl reaction. The PCR program was 94°C for 30 seconds and 72°C for 3 minutes, five cycles; then 94°C for 30 seconds, 70°C for 30 seconds and 72°C for 3 minutes, five cycles; followed by 25 cycles of 94°C for 30 seconds and 68°C for 30 seconds; and concluding with by an extension cycle of 72°C for 3 minutes. Nested PCR products were end sequenced using NGSP1 or NGSP2. The RACE sequences have been submitted to GenBank EST database (accession numbers from EW712308 to EW712635).

## Mapping RACE sequence to the genome

We first use the BLAT alignment tool [44] to compare all the RACE sequence reads to the human genome assembly (hg17, May 2004), and then evaluated the 'fitness scores' of the BLAT output matches using the following formulas:

$$sizeDif = \mathrm{abs}([tEnd - tStart] - [qEnd - qStart]) + \mathrm{abs}(qSize - [qEnd - qStart])$$

$$insertFactor = qNumInsert + tNumInsert$$

$$total = matches + repMatches + misMatches$$

$$badness = (1{,}000 \times misMatches + insertFactor + 3 \times \log[1 + sizeDif])/total$$

$$fitness = 100 - badness \times 0.1$$

Where parameters such as *tEnd* have the same meanings as those defined in the BLAT documentation. The fitness score is based on the 'percent identity score' in the University of California at Santa Cruz Genome Browser [45], and it includes additional penalty on small overall matches. Once these fitness scores have been computed for one RACE experiment, a distribution of these scores was derived based on the characteristics of those BLAT matches that are located on the 'correct' chromosomes, and only those 'correct' matches with scores above a certain threshold were kept as 'valid' products and correspondingly as 'valid' transcripts. See Additional data file 2 for further details.

## Consensus splice site analyses

For those BLAT matches with multiple blocks, the corresponding splice sites in the transcripts were further examined in the following way. A splice site is defined as a consensus one if and only if a 'GT-AG' (or 'GC-AG'/'AT-AC', which appear much less often) pattern can be observed within windows of eight nucleotides on the two ends of it. (For example, for a splice site starting at chromosome position *i* and ending at *j*, the windows are $[i - 3, i + 5)$ and $[j - 5, j + 3]$.) An overall consensus score was then assigned to each transcript according the proportion of consensus splice sites in all its splicing events. We also used this consensus splice site criteria to filter out mirroring antisense transcripts caused by experimental artifacts. See Additional data files 3 for discussions on the choice of window size and the resulting transcripts after this filtering.

## Analyzing the correlation of signal intensity and transcript characteristics

Normalized signal intensities from across tiling array experiments were extracted for those primer regions and correspondingly assigned to the transcripts. These signal intensities were correlated with different transcript characteristics such as splicing events in our analysis.

## Analyzing the connectivity to known exons/novel TARs

The 'valid' transcripts were also compared against RefSeq gene annotation [46] and the union of all of the novel TARs for connectivity information. For each transcript, its connectivity to known RefSeq genes is the number of unique known exons that overlap with this transcript, and the connectivity to novel TARs is the number of novel TARs that overlap with this transcript.

### *Protein homology analysis of the novel transcripts*

We consider a RACE sequence (either a single block one or with consensus splice sites) a 'novel transcript' if it is not connected to any RefSeq genes. We consider it a 'novel isoform' of a known gene if it overlaps with a known gene and has at least 50 bp not covered by existing annotation. We then compared all such novel transcripts to the nonredundant database using BLASTX [27], and selected those transcripts that have at least 50 amino acids significant matches in the database as candidates for further analysis. For the 'novel isoform' sequences, we then examined whether they encode a different protein domain.

### *Real-time RT-PCR*

Human NB4 cell line total RNA or placenta polyA+ RNA (Ambion) were used to make 5'-RACE-Ready cDNA, as described above. Real-time quantitative PCR experiments were performed in quadruplication using LightCycler® 480 Probe Master or TaqMan® Universal PCR Master Mix according to the manufacturer's instructions on a LightCycler® 480 system (Roche Applied Science, Indianapolis, IN, USA). Human *HPRT1* endogenous control, human *SYN3* (Hs00185853_m1), and *TIMP3* (Hs00165949_m1) TaqMan® gene expression assays were ordered from ABI (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR primers for novel TAR RACE product 5NGSP2F8 (left primer: tacagcgcagccagaatg; right primer: gggcaggcaaagtcagataa; ProbeLibrary probe: #87; catalog no. 04689127001) were designed using Universal ProbeLibrary Assay Design Center [47] (Roche Applied Science). Primers were designed across a splice junction. The amplicon is 60 bp. Serial dilutions of cDNA template (400 ng, 100 ng, 25 ng, and 6.25 ng) were used in 20 μl real-time quantitative PCRs. The PCR program parameter was 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, and a final cooling step of 40°C for 10 seconds. The PCR amplification efficiencies among *HPRT1* and our target transcripts are close (within 10%), and *HPRT1* amplification is in the same linear range as our target transcripts. Roche relative quantification software was used to compare the relative expression levels of our target transcripts with *HPRT1*.

### *Direct labeling of total RNA and cDNA and hybridization to ENCODE tiling arrays*

Total RNA and cDNA from human NB4 cells was chemically labelled with biotin using ULS reagent from Kreatech (Amsterdam, The Netherlands) for total RNA and LabelIT reagent from Mirus Bio (Madison, WI, USA) for cDNA. Five micrograms of total RNA and cDNA per array hybridization was incubated with labeling reagent for 30 minutes at 85°C and 60 minutes at 37°C, respectively. Samples were then purified with Qiagen PCR purification columns (Qiagen, Valencia, CA, USA) and ethanol precipitation, respectively. Labelled samples were hybridized to Affymetrix (Santa Clara, CA, USA) ENCODE 1.0 oligonucleotide tiling microarrays. Each sample was hybridized in triplicate to both the forward-strand and reverse-strand version of the array, using the manufacturer's standard hybridization, staining, and washing protocols. The arrays were scanned on an Affymetrix 7G Plus GeneChip scanner, and the signal intensity data were processed using a sliding window of 101 bp.

## Abbreviations

bp, base pairs; ENCODE, ENCyclopedia Of DNA Elements; EST, expressed sequence tag; GSP, gene-specific primer; NGSP, nested gene-specific primer; nonTx region, region not previously shown to be transcribed; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; SAGE, serial analysis of gene expression; TAR, transcriptionally active region; UPM, universal primer A.

## Authors' contributions

Experiments were designed by JQW with suggestions from MS. Experiments were performed by JQW. Bioinformatics analyses were performed by JD; JR and ZZ helped with data analyses. AEU and GE contributed to direct labelling of total RNA. Experiments were performed in the laboratory of MS and SW. Bioinformatics analyses were performed in the laboratory of MG. All authors read and approved the final manuscript.

## Additional data files

The following additional data are available with the online version of this paper. Additional data file 1 shows examples of RACE PCR products on an agarose gel. Additional data file 2 contains example of a histogram of the 'fitness scores' of unique BLAT matches. Additional data file 3 further explains consensus splice site analyses and shows the squared values of probabilities computed. Additional data file 4 contains a file that can be uploaded to University of California at Santa Cruz Genome Brower to view all RACE products.

## Acknowledgements

We thank Janine Mok and Dan Gelperin for critical reading of the manuscript and Jin Lian for NB4 total RNA. We thank Kenneth Nelson and Rajini Haraksingh for technical assistance. We acknowledge the members of the Snyder laboratory for help and support. JQ Wu is supported by NIH Ruth L Kirschstein National Research Service Award and an NIH training grant. M Snyder and M Gerstein are partially supported by the grants from the NIH.

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