

# Genomic analysis of human microRNA transcripts

Harpreet Kaur Saini\*, Sam Griffiths-Jones<sup>†‡</sup>, and Anton James Enright\*<sup>§</sup>

\*Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom; <sup>†</sup>Faculty of Life Sciences, University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, United Kingdom

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**MicroRNAs (miRNAs) are important genetic regulators of development, differentiation, growth, and metabolism. The mammalian genome encodes ≈500 known miRNA genes. Approximately 50% are expressed from non-protein-coding transcripts, whereas the rest are located mostly in the introns of coding genes. Intronic miRNAs are generally transcribed coincidentally with their host genes. However, the nature of the primary transcript of intergenic miRNAs is largely unknown. We have performed a large-scale analysis of transcription start sites, polyadenylation signals, CpG islands, EST data, transcription factor-binding sites, and expression ditag data surrounding intergenic miRNAs in the human genome to improve our understanding of the structure of their primary transcripts. We show that a significant fraction of primary transcripts of intergenic miRNAs are 3–4 kb in length, with clearly defined 5' and 3' boundaries. We provide strong evidence for the complete transcript structure of a small number of human miRNAs.**

**M**icroRNAs (miRNAs) are short (21- to 23-nt) RNAs that bind to the 3' untranslated regions (3' UTRs) of target genes (1, 2). This binding event causes translational repression of the target gene (3) or stimulates rapid degradation of the target transcript (4). miRNAs control the expression of large numbers of genes (5, 6) and are involved in crucial biological processes, including development, differentiation, apoptosis, cell proliferation, and disease (7). Indeed, recent studies have implicated miRNAs in numerous human diseases such as colorectal cancer, chronic lymphocytic leukemia, and Fragile X syndrome (8–12). Expression analyses show that miRNAs are highly and differentially expressed, with specific miRNAs active in specific tissues at specific times (13). In many cancers, miRNA expression is significantly altered, which has been shown to be a useful diagnostic tool (14).

Although our knowledge of miRNA biology has advanced rapidly, attention has been focused on processing and targeting. A miRNA is processed from a longer transcript, referred to as the primary transcript (pri-miRNA) (1, 15, 16). The pri-miRNA includes a precursor miRNA hairpin (pre-miRNA), or sometimes more than one cotranscribed pre-miRNA (16–18). The processed pre-miRNA hairpin is exported to the cytoplasm, where the final mature miRNA is excised (1, 15). Although we know the genomic coordinates of the mature and precursor sequences on the genome, we often know very little about the primary transcript. Approximately 50% of human miRNAs appear to be expressed from introns of protein-coding transcripts (19). However, only a handful of intergenic pri-miRNAs have been characterized. These studies indicate that they can be up to several kilobases long and contain a 5' 7-methylguanosine cap and a 3' polyadenylated [poly(A)] tail (17, 18, 20), implying transcription by RNA polymerase II. Past studies have also identified canonical TATA box motifs upstream of miRNA genes (21). In contrast, a recent study provided evidence for transcription of a few miRNAs by polymerase III, where these miRNAs were found to be interspersed by Alu repeats (22). Full understanding of miRNA transcription requires a complete description of the location and extent of pri-miRNAs, including transcription start sites, promoters, and transcription factor (TF)-binding sites.

Annotation of the primary transcripts of miRNAs is extremely important to our understanding of the biology of miRNAs and their regulatory targets. This information allows (i) the prediction of upstream regulatory regions such as TF-binding sites; (ii)

the detection of other sequence and structural motifs around the miRNA that may be recognized by the processing machinery; (iii) the mapping of SNPs and mutations that may be outside the mature miRNA but affect its transcription or processing; and (iv) provides information required to build targeting constructs for miRNA knockout.

Although previous studies (23, 24) have looked at transcriptional features associated with miRNAs, they focused on individual features in isolation [expressed sequence tags (ESTs) and TF-binding sites]. In this work, we seek to combine multiple sets of features from large-scale genomic data with experimentally validated miRNA data to predict and describe features of pri-miRNA transcripts and to delineate their genomic boundaries. Similar analyses have proved invaluable for coding gene prediction (25). We located intergenic miRNAs on the human genome and mapped the following features to the surrounding regions: transcription start site (TSS) predictions, EST matches, CpG island predictions, gene identification signature–paired-end ditags (GIS-PET) matches, TF-binding site predictions, and poly(A) signal predictions. We found consistent 5' and 3' sequence features outside pre-miRNAs that clearly delineate the boundaries of miRNA transcripts. For many intergenic miRNAs, we predict the extent of their primary transcripts by using these data. Our results indicate that the majority of primary transcripts of intergenic miRNAs are shorter than protein-coding transcripts, with TSSs located within 2,000 bp upstream and poly(A) signals located within 2,000 bp downstream of the pre-miRNA. Additional features such as ESTs, conserved TF-binding sites, and expression ditags provide further insight into the structure of primary transcripts and the transcription of clustered miRNAs as polycistronic transcripts.

## Results and Discussion

We analyzed six sets of data: TSSs, CpG islands, ESTs, TF-binding sites, expression ditags, and poly(A) signal predictions. Each of these features is discussed in detail below.

**TSSs.** We searched 10 kb upstream and downstream of all 474 human miRNAs for TSSs by using the Eponine method (26). At a threshold of 0.990, Eponine predicted TSSs for 203 (43%) miRNAs. The total number of TSS predictions was 5,190; 172 miRNAs had multiple predicted TSSs, whereas 31 miRNAs were found to have only one predicted TSS. The distribution of distances of predicted TSSs from the pre-miRNA is shown (Fig. 1*a*). As expected, TSS

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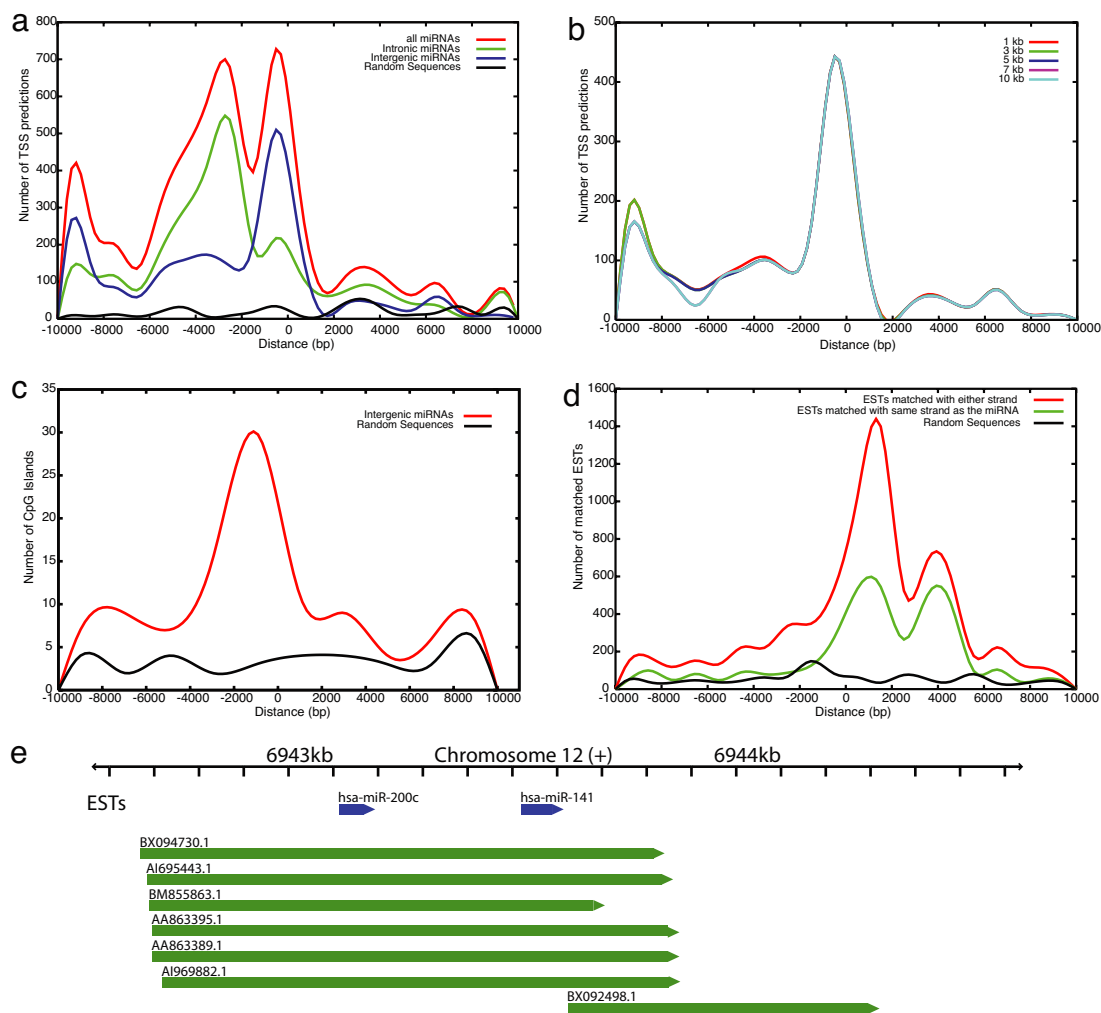
Abbreviations: GIS-PET, gene identification signature-paired end ditags; miRNA, microRNA; poly(A), polyadenylated; pre-miRNA, precursor miRNA; pri-miRNA, primary transcript of microRNA; TF, transcription factor; TSS, transcription start site; UCSC, University of California, Santa Cruz.

<sup>†</sup>To whom correspondence may be addressed. E-mail: sam.griffiths-jones@manchester.ac.uk.

<sup>§</sup>To whom correspondence may be addressed. E-mail: aje@sanger.ac.uk.

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**Fig. 1.** Distribution of TSS, CpG, and EST distances from the 5' end of the pre-miRNAs (positioned at zero) and in random intergenic sequences. (a) TSS predictions. (b) TSS predictions for clustered intergenic pre-miRNAs. (c) CpG predictions. (d) EST predictions. (e) The miRNA cluster hsa-mir-200c~141 overlapped by six ESTs aligned completely in the upstream and downstream regions.

predictions are located almost exclusively in the upstream region. The majority of TSSs occur within 6 kb upstream of the pre-miRNA start. Moreover, this distribution can be divided into three distinct regions: the first lying close to the pre-miRNA within 2 kb upstream and containing 22% of the total TSS predictions; the second broad region from  $-2$  kb to  $-6$  kb, with the highest peak centered on  $-4$  kb comprising 39% of TSSs; and the third region from  $-9$  kb to  $-10$  kb, containing 7% of TSSs. We speculate that the three different regions correspond to different sets of miRNAs. Indeed, intronic and intergenic miRNAs have distinct distributions (Fig. 1a). There are 2,875 and 2,315 TSS predictions for 104 intronic and 99 intergenic miRNAs, respectively. TSS predictions for intronic miRNAs lie predominantly in the region from  $-2$  kb to  $-6$  kb (Fig. 1a). The other two regions contain higher peaks for intergenic miRNAs than the intronic ones. Overall, 36% and 64% of TSS predictions in these regions belong to intronic and intergenic miRNAs, respectively.

It is accepted that intronic miRNAs are generally transcribed along with their host genes (19). Eponine predicted TSSs of intronic miRNAs should therefore correspond with the annotated TSSs of the host transcript. Indeed, we find that the majority of predictions lie within 1 kb of the annotated TSS, and the distribution of distances between predicted and annotated TSSs is peaked  $\approx 0$  [see supporting information (SI) Fig. 3]. The 1-kb variation in position

corresponds well with the published positional accuracy of the Eponine method (26).

Intergenic miRNAs are independent transcriptional units, with their own transcriptional regulatory elements. For such miRNAs, there are two separate and prominent peaks containing predicted TSSs (Fig. 1a). The first lies close to the pre-miRNA (within 2 kb) and contains 31% of the total TSS predictions, whereas the second (approximately  $-10$  kb) lies further from the pre-miRNA and contains 11% of the TSS predictions. We find 15 miRNAs that possess TSSs in the region from  $-9$  kb to  $-10$  kb. Interestingly, all of these miRNAs have more than one predicted TSS, with the majority of predictions lying between  $-8$  kb and  $-10$  kb. No other annotated transcripts are identified in these regions. We conclude that a minority of intergenic pri-miRNAs are long with TSSs centered on  $-10$  kb. As a control, we used Eponine to predict TSSs in randomly selected intergenic sequences of length 20 kb (Fig. 1a). The distribution of predictions in random intergenic sequences differs greatly from that surrounding miRNAs, indicating that the distribution of TSSs at  $-2$  kb and  $-10$  kb of intergenic miRNAs is highly nonrandom.

Many intergenic miRNAs are clustered in the genome, suggesting that more than one pre-miRNA may be processed from the same primary transcript (27). We clustered miRNAs at different distance cutoffs (1–10 kb) and tested whether TSS predictions

support their cotranscription. With an inter-miRNA distance of <1 kb, there are 38 clusters formed by 97 miRNAs. Of 38 clusters, 17 are found to have predicted TSSs within 10 kb upstream. The TSS distribution remains largely unchanged, even at large clustering distances, which suggests that there are very few TSS predictions in the regions between pre-miRNAs within a single cluster. For example, of a total of 17 clusters with predicted TSSs, only 6 clusters have TSS predictions between the clustered miRNAs. We conclude that some miRNAs separated by up to 10 kb are transcribed together in a single primary transcript.

**CpG Islands.** CpG islands (28) are useful aids for promoter prediction because they are known to colocalize with TSSs. We looked for CpG islands within 10 kb upstream and downstream of intergenic miRNAs. In total, 111 CpG islands were identified for 82 intergenic miRNAs. Twenty-one miRNAs were found to possess more than one predicted CpG island, with most of these associated with two CpG islands. One exception to this finding was hsa-mir-9-3, which had five CpG islands. The mean length of predicted CpG islands was 1.4 kb. Furthermore, 25 pre-miRNAs were found to be completely embedded in CpG islands. In a recent study, it was shown that the expression of CpG-embedded miRNAs is regulated by hypo/hypermethylation of their associated CpG islands (29), which can further contribute to their distinct expression profiles in normal and cancerous cells (29, 30). Although CpG islands are frequently associated with constitutively expressed housekeeping genes, their role in regulation of expression of miRNAs is still poorly understood.

The distribution of distances of CpG islands from the start of the pre-miRNA and in random intergenic sequences is shown in Fig. 1c. There is a considerable difference in the distribution plots for CpG islands of miRNAs and random intergenic sequences. CpG islands are identified both upstream and downstream of miRNA sequences, with a significant proportion (40%) overlapping with predicted TSS sites within 4 kb upstream. This colocalization provides compelling evidence that promoters of intergenic miRNAs are often located within 4 kb upstream of the pre-miRNA. Treating clusters of miRNAs (defined at different inter-miRNA distances) as single units does not significantly alter the distribution (see SI Fig. 4).

**ESTs.** We searched for ESTs mapped to within 10 kb of all 249 intergenic miRNAs. A total of 222 miRNAs had 7,014 matching ESTs. Only 55 pre-miRNA sequences were overlapped by 336 ESTs, whereas the remaining 167 had EST matches in their flanking sequences. More ESTs (5,021) matched downstream of the pre-miRNA than upstream (1, 657). Among the miRNAs with matched ESTs, 19 miRNAs had only one EST match, 117 had 2–10 matched ESTs, 20 had 11–20 matched ESTs, and 66 had >20 EST matches. Furthermore, the multiple ESTs matching the flanking sequences of a pre-miRNA exhibited significant overlap, providing excellent evidence for the boundaries of the pri-miRNAs.

The distribution of matched ESTs to the flanking sequences of intergenic miRNAs is shown in Fig. 1d. Most of the EST matches are located between –2 kb and +4.5 kb. There are more ESTs matched in the downstream region, and a small excess match the antisense strand. We note that the strand specificity of ESTs is often poorly determined, but the excess of antisense ESTs matching immediately downstream of pre-miRNAs warrants further investigation. These findings are consistent with a previous study by Gu *et al.* (23) who also reported that  $\approx 76\%$  of the investigated miRNAs have matched ESTs in their upstream 2.5 kb and downstream 4 kb flanking sequences and cover mostly the downstream region of pre-miRNAs.

We also investigated the distribution of matched 5' and 3' ESTs separately. We find that: (i) there are more matches to 3' ESTs (40% of matched ESTs are 3', 31% are 5' ESTs, and the rest are

unannotated); (ii) the majority of 3' ESTs matched in the downstream region, whereas 5' ESTs matched both in the downstream and upstream regions; and (iii) most of the 5' ESTs are matched to the same strand as the pre-miRNAs. ESTs contained within 2 kb upstream of pre-miRNA overlap with the distribution of predicted TSSs and CpG islands (Fig. 1d).

We examined EST support for clusters of miRNAs expressed from a single primary transcript. Different clustering distances do not significantly alter the distribution of EST overlap (see SI Fig. 5), with matched ESTs spanning from –2 kb to +4.5 kb. At the 10-kb clustering distance, there are 34 clusters, of which 30 have matched ESTs. Only five clusters have ESTs that map across the whole cluster (SI Table 3), providing strong evidence that each cluster is transcribed as a single transcript. For instance, the cluster formed by the miRNAs hsa-mir-374~545 on chromosome X is entirely overlapped by 6 ESTs, where the majority are 5' ESTs in the same orientation as the cluster (SI Table 3). Similarly, the cluster on chromosome 12 formed by miRNAs hsa-mir-200c~141 is found to be completely overlapped by 6 ESTs with significant overlap in the upstream and downstream regions (Fig. 1e).

The remaining 25 clusters are found to have ESTs matched only to their flanking sequences or partially overlapping the cluster. We observed that in such instances the flanking ESTs are often perfectly overlapped with each other, defining either the 5' or 3' end of a cluster. Aligned flanking ESTs are useful in demarcating the transcript boundaries and thus estimating the length of the pri-miRNA. For instance, the cluster formed by miRNAs hsa-mir-23a~27a~24-2 has 10 ESTs in its downstream region with a perfect overlap at 1,771 bp from the 3' end of hsa-mir-24-2, defining its 3' boundary, which is in agreement with reported experimental data regarding the 3' end structure of hsa-mir-23a~27a~24-2 (18). Another cluster formed by miRNAs hsa-mir-29b-2 and hsa-mir-29c is found to be overlapped by >20 ESTs aligned with perfect 3' overlap, 333 bp downstream of hsa-mir-29c. Similarly, the cluster formed by hsa-let-7a-1~7f-1~7d has perfect overlap of 3' ends of flanking ESTs at 637 bp downstream of hsa-let-7d.

It is also possible to derive hypotheses regarding the expression profile of miRNAs based on tissue expression data of mapped ESTs. For example, hsa-mir-122a is found to have 26 ESTs matching with its flanking regions, 24 of which are expressed in liver. Previous reports have determined the liver specificity of hsa-mir-122a by detailed and laborious cloning studies (31). Similarly, we are able to derive the tissue expression of skeletal muscle-enriched miRNAs (133b, 206, 143) (32) and brain-specific miRNAs (124a-1, 124a-2, 124a-3) (32) based solely on the flanking matched ESTs.

**TF-Binding Sites.** Generally, TF-binding sites are 8–15 bp long and located in upstream regions near the TSS. We searched for potential TF-binding sites in the flanking regions of clustered and unclustered miRNAs to define their positional preferences and their class representation. TF-binding sites can also be used to locate the core promoters of miRNA genes. To date, only the promoters of two miRNA genes (hsa-mir-23a~27a~24-2 and hsa-mir-371~372~373) are known experimentally (18, 21).

In total, 163 miRNAs were found to contain a total of 731 predicted TF-binding sites for 161 TFs. Of these miRNAs, 29 were found to possess only one predicted TF-binding site, most of which were located in the upstream regions. The maximum number of TF-binding site predictions was obtained for hsa-mir-9-2, with 26 TF-binding sites followed by hsa-mir-125b-1 with 16 sites.

The distribution of TF-binding site distances from pre-miRNAs is shown in Fig. 2a. The flanking regions of miRNAs contain significantly more binding sites than random sequences, and the distribution is highly nonrandom. The distribution of TF-binding sites around miRNAs heavily overlaps with that of predicted TSSs and CpG islands. Several other observations follow from the mapping of TF-binding sites. (i) TF-binding site predictions are enriched in upstream regions (69.6% and 30.4%





**Table 1. Top 5 of 72 TFs identified within 2 kb upstream of human intergenic miRNAs**

TransFac ID	TransFac class	Factor name	Gene ontology	P value
MSX1.01	Homeobox	MSX1, HOX7: Homeobox protein MSX-1	Skeletal development	0.00004
NCX.01	Homeobox	TLX2, HOX11L1, NCX: T cell leukemia homeobox protein 2	Development	0.0047
CDC5.01	Tryptophan clusters	Cell division control protein 5	Regulation of transcription	0.0047
SRF.01	Mads	Serum response factor	RNA polymerase II transcription factor activity; signal transduction	0.0264
RP58.01	Unannotated	Zinc finger protein 238	Negative regulation of transcription from RNA polymerase II	0.0264

searched for four different poly(A) hexamers in the flanking regions of miRNAs by using the dnafsmminer tool (37). The distribution of poly(A) signals is shown in Fig. 2*b*. We identified a total of 1,560 instances of these poly(A) hexamers in 249 intergenic miRNAs. A total of 28 miRNAs had one putative poly(A) signal, 41 miRNAs had two poly(A)s, 22 miRNAs had three poly(A)s, and the remainder had more than four signals. The most frequently occurring hexamers were AATAAA and ATAAAA, comprising 31.7% and 25.3% of the total poly(A) hexamers identified, respectively. Treating clustered miRNAs as single transcriptional units results in a greater proportion of poly(A) predictions falling <1.8 kb downstream of the last miRNA. The mean distance of the first AATAAA was 830 bp from the 3' end of the pre-miRNA. The distribution of poly(A) site predictions in randomly chosen intergenic sequences is also shown (Fig. 2*b*). The number of predictions in random intergenic sequences is higher in regions flanking miRNAs, demonstrating that the specificity of the prediction is unsurprisingly poor. A poly(A) hexanucleotide signal alone is therefore insufficient evidence for a 3' end. However, the peak of poly(A) predictions between 0 and 2 kb downstream of pre-miRNAs remains clearly above that in surrounding regions.

Very few miRNAs have experimentally verified poly(A) tails. Lee *et al.* (18) experimentally characterized the presence of a poly(A) tail at 1,752 bp downstream of hsa-mir-24-2. We also identified a putative poly(A) signal AATAAA with a significant dnafsmminer score (0.939) at 1,752 bp downstream of the 3' end of hsa-mir-24-2 in a cluster formed by hsa-mir-23a~27a~24-2. As discussed above, we also identified >10 ESTs in the downstream region of this cluster, perfectly aligned at 1,771 bp from the 3' end of hsa-mir-24-2. However, no single EST covers the entire cluster. In addition, we found other examples of putative poly(A) signals supported by proximally aligned ESTs. For instance, a cluster

formed by miRNAs hsa-mir-29b-2~29c possesses a poly(A) signal (dnafsmminer score = 0.952) at ~311 bp downstream of the 3' end of hsa-mir-29c, close to the ESTs aligned at ~333 bp.

**GIS-PET.** GIS analysis covalently links the 5' and 3' signatures of each full-length transcript into a paired-end ditag (PET) (38). Ditags provide unique identifiers for transcript 5' and 3' ends and are useful in defining their boundaries (38, 39). The prediction of TSS sites/CpG islands can be supported by mapping the 5' end of ditags or 5' CAGE tags in the upstream region. Similarly, mapping of the 3' end of ditags can confirm the location of poly(A) tails. We identified a few instances where the ditag 5' ends are mapped in the upstream region close to the predicted TSSs or CpG islands (within 100 bp). For example, the cluster of miRNAs hsa-mir-181c~181d is found to contain the 5' end of ditags U\_443297 and U\_166195 at -1,607 bp from the 5' end of hsa-mir-181c overlapping with predicted CpG islands (present at -1,916 bp upstream of hsa-mir-181c). Similarly, many 3' ditags overlap 3' ends of ESTs. For example, the cluster formed by miRNAs, hsa-mir-200c and hsa-mir-141 is found to contain ditags U\_59235 and U\_115145 at 473 bp from the 5' end of hsa-mir-200c and their 3' counterparts present at 230 bp from the 3' end of hsa-mir-141. The 3' ditags support the putative poly(A) signal identified at ~211 bp and overlapping ESTs ending at 250 bp from the 3' end of hsa-mir-141 (Fig. 2*c*). Taken together, these data strongly suggest that the putative 5' and 3' boundaries of cluster hsa-mir-200c~141 are approximately -400 bp and +250 bp, respectively, with primary transcript length of ~1,150 bp.

**Prediction of Putative Boundaries of Pri-miRNAs.** The data presented in this work provide strong evidence for the length and boundaries

**Table 2. High-confidence predictions of boundaries and lengths of primary transcripts of 15 intergenic miRNAs**

miRNA/cluster	Chromosome (strand)	Predicted 5' end of primary transcript	Predicted 3' end of primary transcript	Predicted length of primary transcript, bp	Supporting evidence
hsa-mir-200c~141	12(+)	6942737	6943865	1,128	TSS, CpG, ESTs, ditags, poly(A)
hsa-mir-497~195	17(-)	6861211	6863698	2,487	ESTs, 5' CAGE
hsa-mir-34b~34c	11(+)	110888630	110889820	1,190	TSS, CpG, 5' CAGE, ESTs, poly(A)
hsa-mir-29b-2~29c	1(-)	206041489	206046102	4,613	ESTs, 5' CAGE, ditags, poly(A)
hsa-mir-572	4(+)	10979348	10984460	5,112	TSS, CpG, 5' CAGE, ditags, ESTs, poly(A)
hsa-mir-124a-1	8(-)	9794986	9800634	5,648	TSS, CpG, 5' CAGE, ESTs, poly(A)
hsa-mir-99b~let-7e~125a	19(+)	56885133	56888521	3,388	TSS, CpG, 5' CAGE, ESTs
hsa-mir-424-503	X(-)	133508008	133511322	3,314	TSS, CpG, 5' CAGE, ESTs
hsa-mir-200b~200a~429	1(+)	1088033	1094500	6,467	TSS, CpG, 5' CAGE, ditags, ESTs
hsa-mir-223	X(+)	65152025	65156338	4,313	ESTs, 5' CAGE, poly(A)
hsa-mir-23a~27a~24-2	19(-)	13804510	13808884	4,374	ESTs, ditags
hsa-mir-219-2	9(-)	130193109	130195318	2,209	TSS, CpG, 5' CAGE, ditags, ESTs
hsa-mir-210	11(-)	555660	558587	2,927	TSS, CpG, 5' CAGE, ESTs, poly(A)
hsa-let-7i	12(+)	61283559	61284132	573	TSS, CpG, ESTs, ditags, poly(A)
hsa-mir-92b	1(+)	153431072	153432003	931	TSS, CpG, 5' CAGE, ESTs, ditags

of pri-miRNAs and the transcription of clustered polycistronic miRNAs. Although each feature represents only a small piece of evidence for a transcript end, the combined weight of the diversity and number of analyzed features delineates many 5' and 3' boundaries of pri-miRNAs with high confidence. The 15 best supported pri-miRNAs [by TSS, CpG, EST, ditag, and poly(A) data] are given (Table 2). Detailed graphical views of these and other examples are provided in SI Table 5. It is clear that the length of the primary transcript varies greatly, from 0.5 kb to 7 kb in the 15 examples. In addition, a small set of pri-miRNAs (hsa-mir-374~545, hsa-mir-9-2, hsa-mir-193b~365-1, hsa-mir-181c~181d) appear to have length >10 kb (see SI Table 6). Furthermore, we are able to predict only the 5' or 3' end of the primary transcript of a much larger number of miRNAs (SI Table 7). Based on these results, we derive a canonical structure of the mammalian miRNA primary transcript (Fig. 2*d*) showing the TSS, CpG islands, and overlapping TF sites within 2 kb upstream and poly(A) signal 2 kb downstream of the precursor miRNA.

## Conclusions

Little is known about the structure of pri-miRNA transcripts. Previous studies have focused on individual features (23, 24). In contrast, we present a survey of multiple genomic features of approximately hundreds of annotated miRNAs in the human genome, the most comprehensive analysis of miRNA transcriptional features to date. This analysis has allowed us to delineate the boundaries of a significant proportion of intergenic human miRNAs.

Our results demonstrate that transcriptional features in flanking sequences of miRNA precursors provide strong evidence for the boundaries of pri-miRNAs and for the cotranscription of clustered miRNAs. TSS and CpG island predictions demarcate the 5' ends of many intergenic miRNA transcripts. The data analyzed indicate that a significant fraction of human intergenic miRNAs possess TSS sites within 2 kb of the pre-miRNA. Most CpG islands are found in close proximity to these TSS sites. Poly(A) sites define and

predict 3' boundaries. We show that the distribution of poly(A) signals peaks at 2 kb downstream of the pre-miRNA.

Combining these signal prediction results with EST and expression ditag data provides strong evidence that many human intergenic miRNAs are encoded by primary transcripts 3–4 kb long, with a small fraction of longer transcripts up to 6 kb (Fig. 2*d*). Previous detailed experimental studies of small numbers of pri-miRNAs have also shown transcript lengths of 1–4 kb (17, 18, 20).

Obviously, experimental work is required to determine unambiguously exact pri-miRNA transcript lengths. In contrast, the computational results presented here provide bounds on pri-miRNA transcript lengths, together with a set of high confidence, strongly supported pri-miRNA predictions in an efficient and timely manner. These predictions provide a large-scale look at features surrounding intergenic miRNAs and as such represent a significant step in our understanding of their transcription.

## Materials and Methods

**Obtaining Human Pre-miRNAs.** We obtained genomic coordinates of 474 human pre-miRNAs from the miRBase miRNA sequence database (version 9.0) (40). The human genome sequences and annotations were obtained from Ensembl release 42 (Wellcome Trust Sanger Institute, Cambridge, U.K.) (41) and are clustered according to their genomic distance. For full details, see *SI Materials and Methods*.

**Obtaining Genomic Features.** Flanking sequence data, TSSs (SI Fig. 5), and GIS-PET data were obtained from Ensemble (41, 42). TF-binding sites were obtained using the UCSC browser (34, 43, 44). Poly(A) signals were predicted using the dnafsmimer method. For details, please see *SI Materials and Methods*.

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