

falciparum) (17) and 22923 schyzon (*Cyanidioscyzon merolae*) (18) 5' end clones. These correspond to 3061 zebrafish, 1527 malaria and 3635 schyzon genes. We will continue to expand DBTSS by adding TSS information for other species, such as macaque, when the relevant data become publicly available. Such data will give us a deeper insight into how the transcriptional regulatory networks have been shaped into their current form in humans, in terms of the molecular evolution of the promoters.

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GENOME RESEARCH

Diversification of transcriptional modulation: Large-scale identification and characterization of putative alternative promoters of human genes

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Diversification of transcriptional modulation: Large-scale identification and characterization of putative alternative promoters of human genes

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By analyzing 1,780,295 5'-end sequences of human full-length cDNAs derived from 164 kinds of oligo-cap cDNA libraries, we identified 269,774 independent positions of transcriptional start sites (TSSs) for 14,628 human RefSeq genes. These TSSs were clustered into 30,964 clusters that were separated from each other by more than 500 bp and thus are very likely to constitute mutually distinct alternative promoters. To our surprise, at least 7674 (52%) human RefSeq genes were subject to regulation by putative alternative promoters (PAPs). On average, there were 3.1 PAPs per gene, with the composition of one CpG-island-containing promoter per 2.6 CpG-less promoters. In 17% of the PAP-containing loci, tissue-specific use of the PAPs was observed. The richest tissue sources of the tissue-specific PAPs were testis and brain. It was also intriguing that the PAP-containing promoters were enriched in the genes encoding signal transduction-related proteins and were rarer in the genes encoding extracellular proteins, possibly reflecting the varied functional requirement for and the restricted expression of those categories of genes, respectively. The patterns of the first exons were highly diverse as well. On average, there were 7.7 different splicing types of first exons per locus partly produced by the PAPs, suggesting that a wide variety of transcripts can be achieved by this mechanism. Our findings suggest that use of alternate promoters and consequent alternative use of first exons should play a pivotal role in generating the complexity required for the highly elaborated molecular systems in humans.

[Supplemental material is available online at www.genome.org. The sequence data from this study have been submitted to DDB] under accession nos. DA000001-DA999999, DB000001-DB294747, DB294748-DB384947, BP192706-BP383670, AU279383-AU280837, and AU116788-UI60826.]

One of the most striking findings revealed by the Human Genome Project is that the human genome contains only 20,000–25,000 kinds of protein-coding genes (International Human Ge-

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nome Sequencing Consortium 2004). This number is unexpectedly small compared with the total gene numbers in yeast, fly, and worm genomes, which are estimated to be 6,000, 14,000, and 19,000, respectively (Goffeau et al. 1996; *C. elegans* Sequencing Consortium 1998; Adams et al. 2000). It is supposed that there must be other factors in addition to mere gene numbers to satisfy the prerequisites that enable the human genome to fabricate such highly elaborated systems as the brain and immune systems. To explain this, it has been hypothesized that multifaceted use of the genes should play a pivotal role in functional

diversification of human genes without affecting the total gene number (Ewing and Green 2000).

Multifaceted use of the genes would be enabled either by the production of slightly different transcripts, which are finely tuned for specific purposes from a single gene locus, or by employing essentially the same transcript in different circumstances, or by the combination of these mechanisms. As for the first possibility, recent reports showed that alternative splicing (AS) is employed in about half of all human genes, producing more than three different transcript variants per locus on average (Lander et al. 2001). Various transcripts produced by AS are consequently translated into proteins with slightly different structures and functions, and thus this mechanism is thought to provide a molecular mechanism for the fine tuning of the gene functions of a single locus (Lopez 1998; Black 2000). As for the second possibility, the use of alternative promoters (APs) has been presumed. By utilizing APs, which consist of different modules of transcriptional regulatory elements, diversified transcriptional regulation should be enabled at a single locus (Landry et al. 2003). Combinatory use of these two possibilities (AS and APs) would even further increase the potential complexity of the products expressed from a single gene; for example, multiple separated promoters might independently direct transcription from different genomic positions and the subsequent variation in the first exons might result in the production of N-terminally different proteins.

Actually, for some human genes of particular interest, *in vitro* and *in vivo* experiments have verified that such complex diversification takes place within a cell. For example, the *SHC1* gene has two APs and produces three different transcripts encoding protein isoforms of 46, 52, and 66 kD (Luzi et al. 2000). The transcript encoding p46/p52 is transcribed from the proximal promoter with a ubiquitous expression pattern. On the other hand, the transcript encoding p66, whose biological functions are completely different from those of p46/p52 because of the presence of one additional collagen homology domain at its N terminus, is driven by a distal promoter and is specifically expressed in limited types of cells. The promoters of these two isoforms are approximately 4 kb apart from each other and the repertoires of the predicted potential *cis*-acting regulatory elements are completely different. In addition, recent studies also demonstrated that the histone acetylation and cytosine methylation statuses are significantly different between the two APs (Ventura et al. 2002).

Although our understanding of the comprehensive features of AS has been rapidly advancing with the compiling of EST data (Lee et al. 2003; <http://www.bioinformatics.ucla.edu/ASAP/>), very little is understood about the genome-wide features of the APs so far. Indeed, in spite of increasing general interest and need, almost no reports or databases have provided a genome-wide view of which population of human genes is regulated by APs and what biological consequences such diversification of the transcriptional modulation would bring about. In our opinion, this is because of a general lack of information about the transcriptional start sites (TSSs) and adjacent putative promoter regions (PPRs). For systematic identification of the APs, highly redundant sequence data would be essential. However, the coverage of the EST data at the 5'-ends has generally been low, since the conventional cDNAs are constructed utilizing the 3'-end poly(A) without any selection method for the opposite end, the 5'-end cap structure (Suzuki et al. 1997). Besides, even if available cDNA sequences have already covered the corresponding re-

gions, it cannot be assured that their 5'-ends correspond to the real TSSs without in-depth analysis. For these reasons, the massively accumulated current EST data could not be directly used for the identification and analyses of the APs. Although a pioneering study was done by Zavolan et al. (2003) making use of full-length cDNAs, their data were limited to about 60,000 mouse cDNAs.

We have been collecting full-length cDNAs of human genes by constructing cDNA libraries using the oligo-capping method (Suzuki and Sugano 2003; Ota et al. 2004). Using the full-length cDNA sequence data, we have also demonstrated that the exact positions of the TSSs and the adjacent putative promoters could be retrieved from the human genomic sequences in a high-throughput manner (Suzuki et al. 2001b). Our human full-length cDNA data accumulated so far and the retrieved adjacent upstream PPR information are integrated in our database, DBTSS (Suzuki et al. 2004; <http://dbtss.hgc.jp>) and have been made publicly available now. In this study, we further expand our 5'-end sequence data up to 1.8 million cDNAs (all of the sequence data have been registered in DDBJ and the physical cDNA clones are available on request). With the increase of the entire dbEST data (6 million entries as of March, 2005) by about 30%, these data extensively complement the 5'-ends in the current EST collection. By using our new 5'-end oligo-cap cDNA data, we were able to glimpse for the first time an overview of how the TSSs are clustered and in what manner the adjacent putative promoters are used multifacetedly in humans. Here we report our first genome-wide analysis of the alternative use of putative promoters using our unprecedented collection of 5'-end cDNA data.

Results and Discussion

Massive identification of the transcriptional start sites using 1.8 million 5'-end sequences of putative full-length cDNAs

We computationally mapped 1,780,295 5'-end sequences of putative full-length cDNAs onto the human genome (hg_17 of UCSC Genome Browser; Hsu et al. 2005; <http://genome.ucsc.edu/>). The cDNAs were collected from full-length-enriched cDNA libraries constructed from 164 kinds of human tissues and cultured cells using our cap selection method, the "oligo-capping" (for further details of the protocol for the library construction, see Suzuki and Sugano 2003). The library information is summarized in Supplemental Table 1. The number of cDNA sequences that were successfully mapped from their first base was 1,536,604. Using the positional information of the exactly and uniquely mapped 5'-ends of the oligo-cap cDNAs, we defined the TSSs as the independent genomic positions to which the first bases of the oligo-cap cDNAs were mapped (Fig. 1).

Before generating the final TSS data set, we excluded those cDNA sequences that had been mapped inside the second or later exons of any other cDNAs or RefSeq sequences, which represent previously characterized transcripts (Pruitt et al. 2005; <http://www.ncbi.nlm.nih.gov/RefSeq/>). This was intended to minimize dubious identification of TSSs due to erroneously cloned truncated cDNAs. Although it is relatively minor, our "oligo-cap" cDNA collection does include a certain population of those erroneously cloned species (see also Suzuki et al. 2001a). We presumed that those cDNAs whose 5'-ends were located outside of the exonic regions of any other clones could not be truncated forms of any known types of transcripts, at least. This presump-

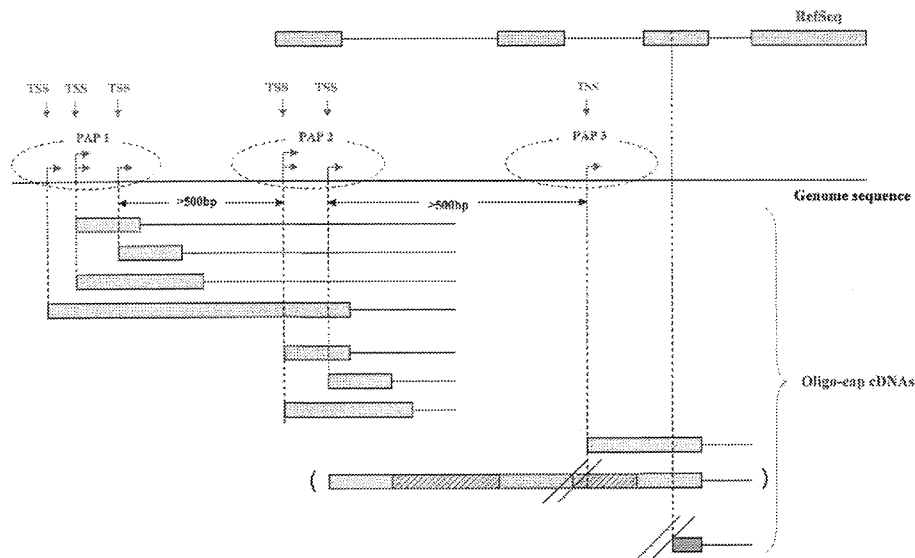


Figure 1. Identification of the putative alternative promoters in human genes. Schematic representation of the mapping of the 5'-ends of the oligo-cap cDNAs, the determination of the TSSs, and clustering of the TSSs to identify the PAPs. The boxes and lines represent exons and introns, respectively. The RefSeq sequences and the oligo-cap cDNAs are in red and blue, respectively. The lowest gray oligo-cap cDNA is excluded from the data set, since its 5'-end is located within an internal exon of the RefSeq. The third-lowest oligo-cap cDNA is accepted because the truncation of the erroneously sliced second-lowest transcript would otherwise need to be hypothesized to explain its presence, and the chance of the combination of such events should be low. The shaded boxes represent the retained introns. Altogether, this case consists of 8 "full-length" oligo-cap cDNAs that are mapped at 6 TSSs, clustered into 3 PAPs.

tion is based on the fact that the combination of multiple errors, for example, truncation followed by erroneous oligo-capping that occurred on an immaturely spliced form, would be required to erroneously generate such cDNAs (see example in Fig. 1). Because the expected rate of such tandem errors should be very small, we considered that most of the selected 5'-ends of the oligo-cap cDNAs should correspond to actual TSSs *in vivo* and that such selection should be helpful to reduce the false-positive identification of TSSs. We did not exclude the TSSs that mapped in the first exons because it has been shown that the locations of TSSs fluctuate to some extent in most genes (Suzuki et al. 2001a). The probability that a truncation event occurred within the typically relatively short first-exonic region and that spurious oligo-capping occurred on the same transcript can be considered to be low, and thus we considered that most TSSs that mapped in first exons should be the result of TSS fluctuations and not of truncated forms.

As a result, we obtained a data set of 1,355,367 TSSs. Given this number, the overall frequency of the full-length cDNAs could be estimated as 88%, which is closely consistent with our previous evaluations of the full-length-ness of the libraries. Furthermore, we validated the accuracy of the TSSs identification by directly comparing the positions of the previously reported TSSs in Eukaryotic Promoter Database (EPD Release 82; <http://www.epd.isb-sib.ch/>; Schmid et al. 2004) with those identified in the present study. As shown in Figure 2, at least 86% (155/181) of the positional information of the TSSs previously detected using different methods [92% (1056/1144) if the TSSs determined by high-throughput methods were also summed] were consistent with our results. Although much more analysis would need to be done, including exhaustive RACE analyses, before we could actually conclude that all of the TSSs identified in this study cor-

respond to real TSSs from which transcription was truly initiated *in vivo*, we consider that the error rate of the current data set should be sufficiently low to glimpse the general features of the APs via the analysis presented below.

Among the identified TSSs, 1,171,916 TSSs corresponded to RefSeq genes (LocusLink locus; now superseded by Entrez Genes) (Maglott et al. 2005; <http://www.ncbi.nih.gov/entrez/query.fcgi?db=gene>). Those TSSs collectively covered 14,628 RefSeq genes, that is, 85% of all, RefSeq genes (17,217 genes). The base composition of the exact site of the TSSs was 51%, 25%, 17%, and 7% for A, G, C, and T, respectively, which is consistent with our previous observations using a smaller data set (Suzuki et al. 2001a). Interestingly, the base composition of the 183,415 TSSs that did not correspond to RefSeq genes was significantly different: 41% (A), 17% (G), 17% (C), and 24% (T). Especially notably, the frequency of T was more than three times higher than in RefSeq genes. Assuming that the major part of the protein-coding genes are covered by RefSeq genes and the non-protein-coding transcripts are enriched in the noncovered population, the above observation might imply that the nature of the transcriptional initiation mechanism is different between protein-coding and nonprotein-coding RNAs. In this study, we focused on the TSSs of RefSeq genes for the analyses described below, although it would also be interesting to analyze the nature of this emerging class of non-protein coding RNAs further (Okazaki et al. 2002).

More than half of human genes are regulated by alternative promoters

To identify possible AP-containing genes, we clustered the TSSs of RefSeq genes by binning them by >500-bp intervals (Fig. 1). We tentatively employed this criterion because our previous studies showed that the range over which the TSSs are scattered is on average 62 bp, with a standard deviation of 20 bp (Suzuki et al. 2001a), and thus the chance that TSSs belonging to a single cluster would be scattered over a range of >500 bp would be very low. Also, the statistics shown in Supplemental Figures 1 and 2 support the notion that a 500-bp interval is a strict enough parameter to make a meaningful separation of the clusters of TSSs while at the same time avoiding the erroneous separation of single TSS clusters into multiples. Besides, >500-bp intervals would allow us to hypothesize that the identified putative alternative promoters (PAPs) should not share most of the surrounding sequence context of the TSSs and the major part of the transcriptional regulatory modules, which constitute the direct docking platform of the general transcription factors and the RNA polymerase complex, and the immediate upstream sequences, to which other transcription regulatory factors are most likely to be recruited.

The clustering analyses revealed that a total of 269,774 different positions of TSSs were clustered into 30,964 groups. Each

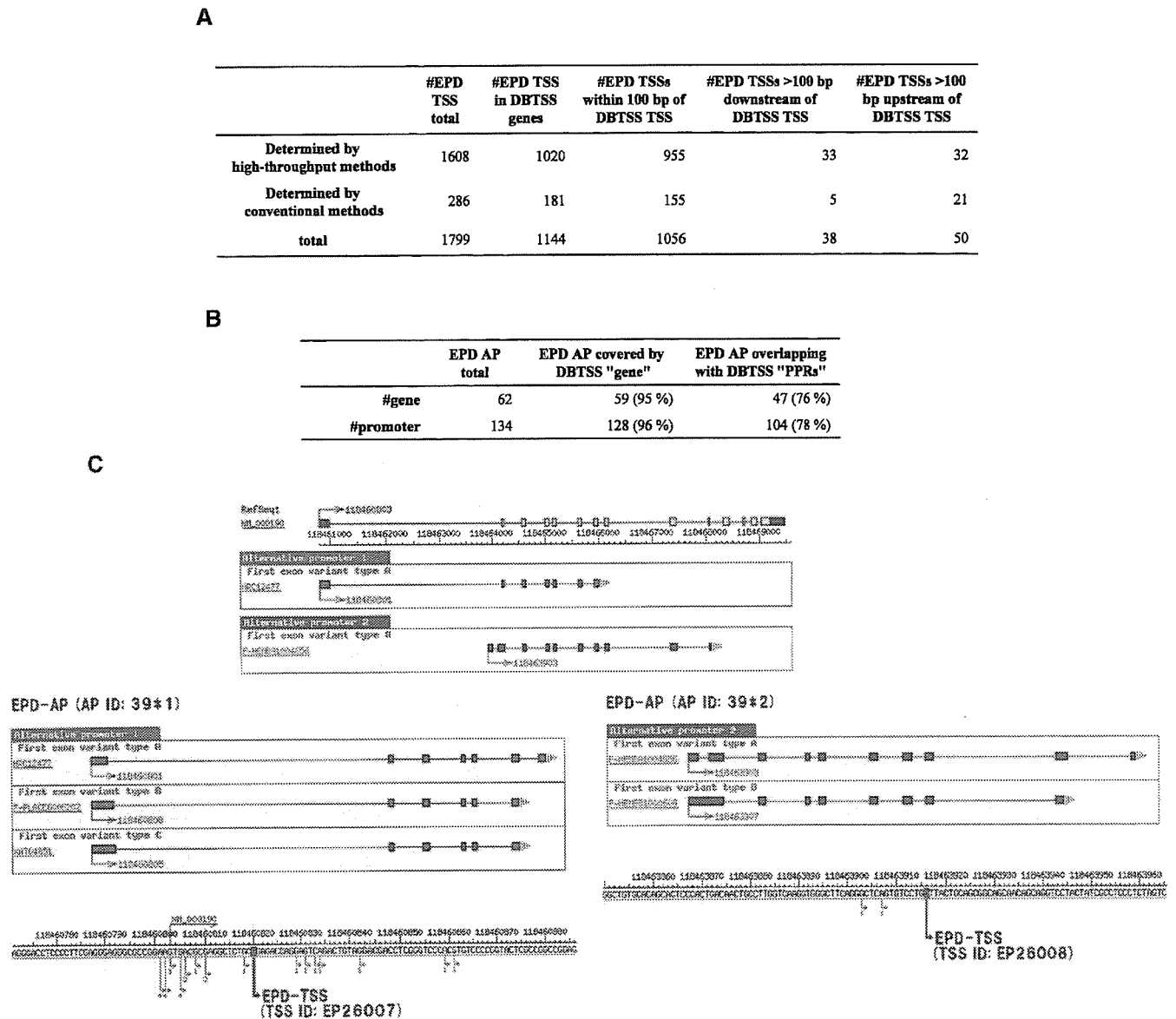


Figure 2. Comparison of the DBTSS data with the previously characterized TSSs and APs. TSSs (A) and APs (B) identified by the DBTSS data were compared with those characterized in previous studies. When a TSS/AP registered in EPD was located within 100 bp of one in DBTSS, they were counted as "overlapping." The margin of 100 bp was allowed considering fluctuations of the TSSs (Suzuki et al. 2001a). (A) The "overlapping" was counted separately for the TSS data obtained from high-throughput cDNA cloning methods (like ours) and that from conventional methods, such as RACE and nuclease protection assays. Note that as some of the TSSs were identified by multiple methods, the total numbers in the third line are not always the sum of the above two. (B) First column is the total number of EPD genes registered as "alternative promoter-containing genes" and the number of the corresponding promoters; second column is the coverage of the DBTSS against EPD at the gene level; third column is coverage of the DBTSS against EPD at the promoter level (all APs were covered by DBTSS PPRs). (C) The case in which EPD data and DBTSS data were overlapping with each other is exemplified by the case of the human hydroxymethylbilane synthase gene (NM_000190). RefSeq exons are shown in blue (non-coding regions) and yellow (coding regions) boxes and the DBTSS exons are shown in red (PAP group 1) and green (PAP group 2) boxes. The lower panels are magnifications of the upper panel(s). The TSSs are represented by arrows of the corresponding colors. The IDs of corresponding EPD data are shown. Note that there are variations in the first exon patterns even within the same PAP group (alternative donor in PAP1 and retaining intron in PAP2) and the TSSs are fluctuating. For additional examples, see Supplemental Table 3.

cluster identified was considered to represent a PPR, and for gene loci with multiple PPRs, each individual PPR was designated a PAP. (Note that they are "putative" promoters, because our TSS determination is mostly based on a single method, oligo-capping, and thus confirmation using other methods as well as functional analyses of the promoters remains essential).

To our surprise, 7674 (52%) of the RefSeq genes contained more than a single TSS cluster (24,010 clusters in total), which

may be regulated by distinct APs. If any, there were 3.1 distinctive PAPs per gene, on average. By way of comparison, genes having alternative splicing forms are thought to constitute 60% of human genes and, if any, there are about 3 AS variants per locus (Lander et al. 2001; Modrek and Lee 2002). (Note that the EST data set from which the AS variants were identified did not include the majority of the new data used in the present study. The number of available cDNAs at the time the AS variant studies

were performed was about 2 million, which is almost the same as the number in the currently used data set.) Our analysis indicates that alternative promoters occur at least with the same order of frequency as the AS variants (see also the section on first exon patterns below). The regulation of transcription thus appears to be more dynamic than previously thought. Although it has been reported that splicing events are highly variable in human genes, this is the first report showing that alternative transcriptional regulation is about equally prevalent in human genes. All of the information produced in this study has been integrated in our database, DBTSS, and the raw data have been made freely downloadable for anonymous public users.

We also examined how many of the previously characterized APs were represented in our newly generated PAP data set. As shown in Figure 2B, there were 134 APs in 62 genes registered in the latest version of EPD. Among them, 104 promoters (78%) in 47 genes (76%) were correctly represented in our data set. Figure 2C shows a case in which the identified APs were supported by both EPD and the present DBTSS data. Additional examples are presented in Supplemental Table 3, in which some of the PAPs were consistent with the EPD data and others were unique in our data set. Based on the high coverage [about 95% (59/62 and 128/134) at the gene and promoter levels] and the consistency [about 80% (47/59 and 104/128) at the gene and promoter levels] with the previous data, we consider that our PAP data should be a useful resource for exploring the genome-wide features of the APs, providing accurate representations of the APs with high reliability of each entry.

Although the frequency of the PAPs was unexpectedly large, the estimated frequency should be near the lower boundary of the actual complexity. First, although we used an unprecedented amount of TSS data (80 TSSs per gene on average, which led to the identification of 3.1 PPRs; see Table 1), additional PAPs could remain undiscovered. Actually, the PAPs used with very low efficiency or PAPs used in very limited cell types might not be included in our data set. Secondly, the procedure of clustering the TSSs with the 500-bp bin might have resulted in a number of falsely clustered distinct PAPs. Lastly and most importantly, to exclude possible truncated cDNAs, we had to neglect the possibility that some of the TSSs are really located within the internal exons of other variant transcripts in this study. Indeed, among the 24 EPD promoters that corresponded to DBTSS PAP-containing genes but did not overlap with the DBTSS PAPs (Fig. 2B), 4 were located in internal positions of the second or later exons. Although they should correspond to genuine APs, they were removed from our data set in exchange for increasing the

reliability of the data. We were concerned that the error rate (frequency of truncated cDNAs) should be much higher among those cDNAs whose 5'-ends were mapped internal exonic regions than those cDNAs that were used in the present study. Unlike in the latter group, only single-step errors (erroneous oligo-capping on the truncated mRNAs) would be accounted for dubious generation of the truncated cDNAs in the former group (see Fig. 1, compare the bottom two lines), which we estimated to occur by the frequency of 10%–15%. Therefore, for this purpose, we considered extensive filtration should be indispensable (see Supplemental Fig. 5). In spite of the technical difficulty, further detailed analyses focusing on this subject should be especially fruitful, since in such "internal" PPRs, the corresponding genomic regions should serve as both exons and promoters, and thus should have several features different from other canonical promoters.

Characterization of the identified PAPs

To study the biological implications of the presence of so many PAPs embedded in the human genome, we first examined the differences between the PAP-containing (i.e., with ≥ 2 PPRs) promoters and the PAP-less (i.e., with only 1 PPR) promoters with respect to the frequencies of basic promoter elements, namely, TATA boxes and CpG islands. As shown in Figure 3, the frequencies of the TATA box-containing (Fig. 2B) and the CpG island-containing (Fig. 3A) promoters were 17% and 58% for the PAP-less promoters, respectively. While the frequency of the TATA box-containing promoters was about the same in the PAP-containing population (11%), the frequency of the CpG island-containing promoters was less than half of that in the PAP-less population (28%). We further examined the ratio of the CpG-containing to CpG-less promoters as related to the number of PAPs. As shown in Figure 3A, the number of CpG-containing promoters per locus did not increase at all as the number of PAPs increased, indicating that the probability of the occurrence of the CpG islands was not proportional to the total number of PAPs. In contrast, the population of CpG-less promoters did increase as the number of PAPs increased. We previously showed that CpG-containing promoters predominantly consisted of promoters whose expression was ubiquitous (Yamashita et al. 2005). It may be generally true of the PAPs that there is one (or a few) ubiquitously expressed promoter(s), accompanied by, on average, 2.6 other promoters used for tissue-specific or signal-dependent expression.

Identification of tissue-specific PAPs

Having observed the above results, we examined whether some of the PAPs appear to contribute to different usage of the genes depending on the tissue types in which they are expressed. Since none of the cDNA libraries used in the present study were produced by a normalization method, it was possible to count the number of the represented cDNA clones within the library for the first approximation of their expression levels. To examine the tissue-specific use of a PAP, the sources of the cDNA libraries of all of the TSSs belonging to a given locus were noted and the information about which tissue they were derived from was collected. The probability of the enrichment of any particular tissue type as a source for cDNAs for a given PAP was judged by considering whether the tissue-source composition of the TSSs belonging to that PAP was skewed compared with the overall tissue-source composition of the TSSs belonging to the same locus. For this, we calculated the hypergeometric distribution of the component TSSs for each of the PAPs. After the Bonferroni

Table 1. Distribution of the putative alternative promoters

No. PAPs	No. locus	No. included TSS positions	No. cDNA clones (avg.)
1 (PAP-less)	6954 (48%)	70,175	43
2	3724 (26%)	67,846	83
3	1821 (12%)	44,455	115
4	1003 (7%)	32,582	160
5	490 (3%)	19,962	166
6	294 (2%)	13,937	159
7	147 (1%)	7948	184
8	85 (0.6%)	4912	194
9	42 (0.3%)	2167	163
10	25 (0.2%)	1650	164
>10	43 (0.3%)	4140	341
total	14,628	269,774	80

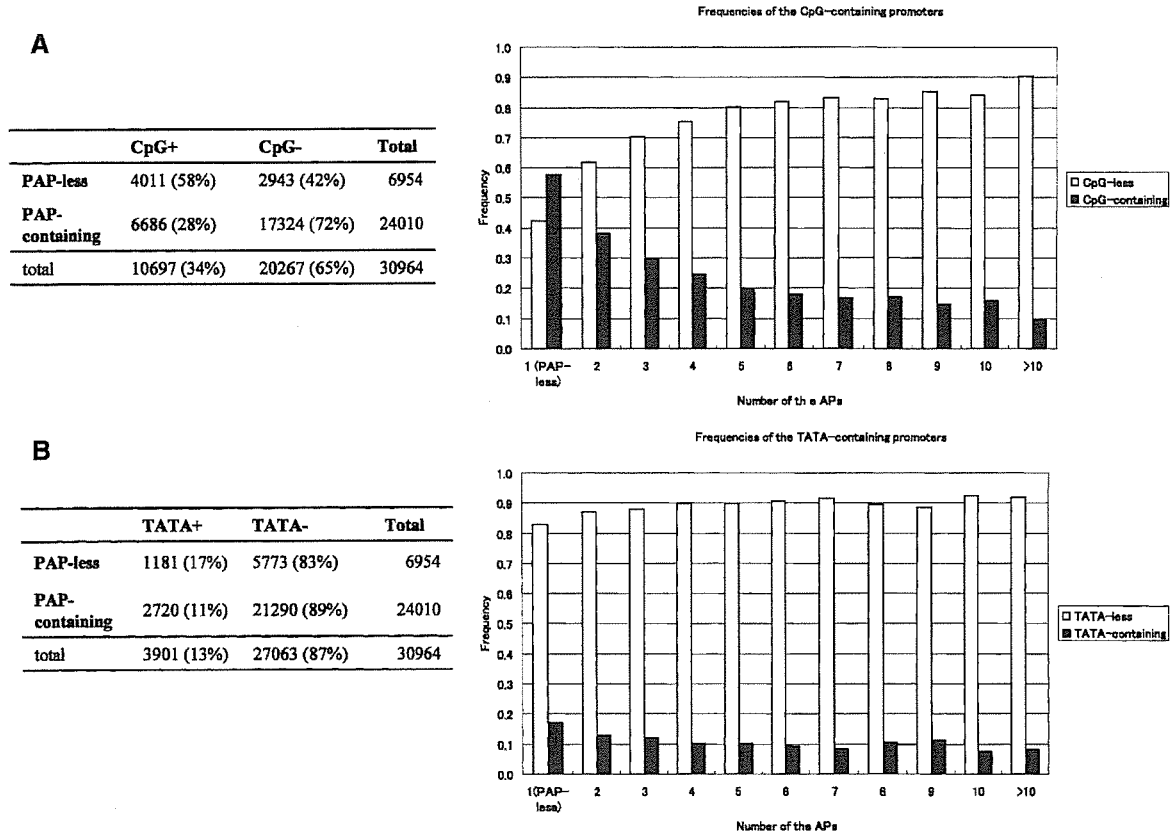


Figure 3. Relationship between the PAPs and the CpG islands and TATA boxes. Frequencies of the CpG island (A) and TATA box (B) containing PPRs. In the right panels, the relationship between the number of PAPs (x-axis) and the frequency of the corresponding promoter motif (y-axis) is shown.

correction, the hits with $P < 0.01$ were selected. The statistical procedure and the subsequent correction were designed so that the statistical bias depending on the coverage of the cDNA libraries (number of the cDNAs sequenced from the library) should be minimized.

Among the PAPs in 7674 PAP-containing loci, 1803 PAPs in 1333 loci (17%) met the above criteria and were designated as "tissue-specific PAPs" (Fig. 4). This indicates that one of the important potential roles of the PAPs should be independent regulation of a gene in a particular tissue-type, while the same gene is subject to different types of regulation in different cellular contexts. The tissue in which such specific usage of the PAPs was most frequently observed was testis tissue. It has been reported that the transcriptional regulation in testis is significantly different from that in other normal tissues. The chromosome condensation and methylation status of the genome are reported to be very special in testis. Actually, many of the antigens specifically expressed during the course of tumorigenesis are expressed in testis under normal conditions, and thus are so-called cancer-testis antigens (Maio et al. 2003; Kalejs and Erenpreisa 2005). The fact that testis was identified as the richest source of the PAPs may indicate that the development of the exclusive use of some types of transcriptional modulation has been indispensable to realizing the functional molecular systems from the heavily skewed genomic structures in germ-line cells. It was also intriguing that the second-richest origin of the "tissue-specific" PAPs was brain. The specific diversification of a large population of genes should also be indispensable for the fabrication of such an

elaborate system as the brain. Considering that the largest populations of the tissue-specific PAPs were related to testis and brain, which separate human species from other organisms most distinctively, it seems likely that further detailed analyses of the PAPs from the molecular evolutionary viewpoint would eventually lead to the identification of genes whose diversification has collectively provided a molecular basis for unique features in humans.

GO classification of the PAP-containing genes

We further investigated in what kinds of genes and with what frequency the alternative use of the first exons was likely to result in alteration of the encoded protein sequences, and what biological consequences it would bring about. We first categorized the PAP-containing genes according to the GeneOntology (GO) classification (Harris et al. 2004; <http://www.geneontology.org/>). The distribution of the GO categories was then subjected to hypergeometric distribution analysis. As shown in Table 2, GO categories of "ATP binding," "phosphorylation," and "kinase" were significantly enriched. The fact that PAP-containing genes were especially enriched in genes for these so-called signal transduction molecules seemed reasonable, since complex transcriptional regulation should be indispensable for appropriate transmission of signals depending on the dynamically changing conditions of the cells.

In contrast, the GO category 'extracellular' was reduced. For extracellular secretory proteins, there are protein-sorting motifs, called "signal peptides," at the N terminus. Because of this re-

A

Rank	Tissue	#Tissue-specific APs	#Locus	Ratio
1	Testis	416	408	0.24
2	Brain	268	261	0.15
3	Immune cells	84	81	0.05
Total		1,731	1,287	

B

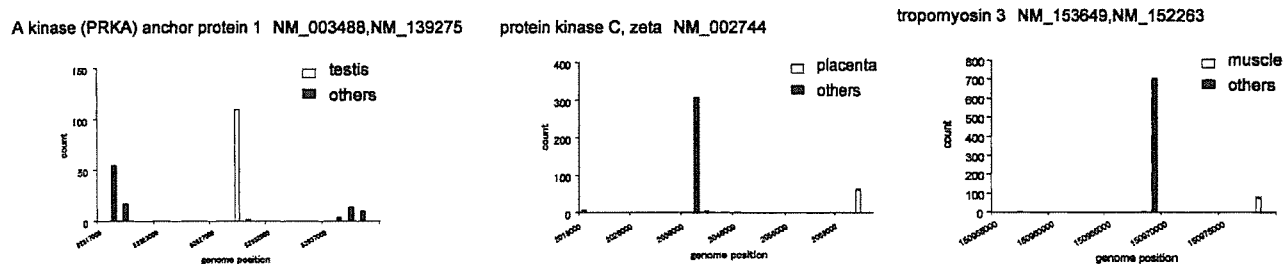


Figure 4. Tissue-specific usage of PAPs. (A) The number of PAPs that are used in a tissue-specific manner. For the detailed definition of the tissue specificity, see the Methods section. (B) Examples of tissue-specific PAPs. The x-axes represent the genomic positions and the bars represent the number of 5'-ends of the oligo-cap cDNAs mapped at the corresponding genomic positions (TSSs). White bars show the tissue-specific usage of the corresponding PAPs observed in the indicated tissues.

striction, the transcriptional regulation could not have freely diverged. It was also intriguing that the G-protein coupled receptors (GPCRs) were infrequent in the PAP-containing genes. Many of the GPCR families are supposed to have been generated via multiple rounds of duplication of the coding exons (Lander et al. 2001; Fredriksson et al. 2003). At least immediately after such gene duplications, it can be assumed that the degree of freedom in the genes themselves should have been relatively high. In such circumstances, transcriptional diversification could have been realized by alteration of the independent locus as a whole for different purposes, and this might account for the lower frequency of the PAPs.

Variation in the first exon patterns contributed by the PAPs and its influence on the downstream protein-coding regions

We also analyzed the first exon sequences to investigate the direct downstream consequences of the PAPs. The patterns of the first exons were classified according to the criteria shown in Figure 5. Indeed, the patterns of the first exons were highly variable as well, partly reflecting the presence of the complex PAPs. In

addition, several patterns of first exon variations were observed for a given PPR. In total, even if 199,599 "TSS variables," which were derived from differing TSSs, were excluded, 54,302 different patterns of the first exon were observed. This means that, on average, there were 7.7 first exon variations and among them 3.1 variants were responsible for separating the PAP groups. Considering that the overall frequency of the hitherto identified AS variants, whose coverage is mostly concentrated in the later exons, is about 3 variants per locus in total, our findings suggest that the first exons are even more variable than the later exons combined.

As shown in Figure 5A, "mutually exclusive" was the most abundant type of first exon. This pattern was responsible for the largest population of the PAPs according to the criteria employed in this study. Although it is supposed that some of the variations belonging to the other categories such as "TSS variable" and "staggered" are actually derived from PAPs that are separated by ≤ 500 bp, this observation lessened the concern that technical errors had led to misidentification of the PAPs, since it is relatively simple to confirm this pattern both empirically and computationally. It should also be noted that "mutually exclusive" variations were more abundantly observed in the first exons than in the later exons. Among hitherto registered AS variants, the population of "mutually exclusive" variations accounted for only several percent (Modrek and Lee 2002; Imanishi et al. 2004). The first exons should have been relatively free from functional requirements to maintain the consistency of the open reading frames or the proper folding of the main parts of the proteins. Because of these characteristics of the

Table 2. Relationship between putative alternative promoters and the GO categories

GO id	GO term	No. locus containing APs	P-value
<i>Enriched</i>			
GO:0005524	ATP binding	678	6e-14
GO:0006468	protein amino acid phosphorylation	325	3e-09
GO:0005856	cytoskeleton	164	2e-08
GO:0004674	protein serine/threonine kinase activity	228	1e-07
GO:0007242	intracellular signaling cascade	205	2e-07
<i>Reduced</i>			
GO:0003735	structural constituent of ribosome	37	4.7e-16
GO:0005576	extracellular region	113	3e-10
GO:0007186	G-protein coupled receptor protein signaling pathway	104	9e-06
GO:0005739	mitochondrion	215	1e-04

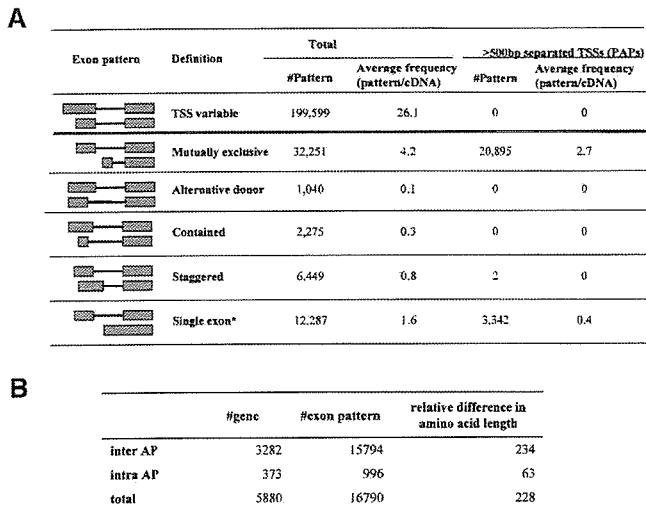


Figure 5. Patterns of the first exons in the PAP-containing genes. (A) Distributions of the patterns of the first exons are shown. The number of identified exon patterns was counted in total (third column) or between the populations which are separated by >500 bp, thus accounting for the separation of the PAPs. *Either of the first exon variations was a "single exon" transcript. Different criteria were employed for them because these transcripts cannot be regarded as "splicing" variants. (B) Alterations of the amino acids resulting from the exon variations occurring in the population of "inter-APs" (TSS distance >500) or "intra-APs" (TSS distance ≤500) were counted.

terminal exons, the occurrence of the "mutually exclusive variations," in which the degree of the freedom is higher than that of the other exon patterns, could have been permitted very frequently during evolution.

As shown in Figure 5B, the alternative use of the first exons following the PAPs caused alterations of the protein coding sequences (CDSs: the positions of the CDSs were defined according to the RefSeq information) in 3282 genes (43% of the 7674 PAP-containing genes), including 15,794 patterns in total. The average length of the alteration in amino acid length was 234 amino acids (a.a). Interestingly, even when no first exon variations changing the CDS were observed between different PAPs (inter PAPs), variations occurring within the same PAPs (intra PAPs) were observed to change the CDS in 373 genes (5%), including 996 patterns in total. Although their influence on the CDSs was relatively small (63 a.a. on average), it is possible that such slight changes also have biological consequences. A short stretch of amino acids could sufficiently mask an N-terminally located motif such as a secretion signal peptide.

Even alterations that did not change the CDS should not be overlooked. In these regions, various sequence motifs involved in modulation of the translational efficiency have been shown to be embedded (e.g., see UTRdb; Mignone et al. 2005). Actually not a few examples have been reported in which very small changes in the first exons play pivotal roles in controlling gene functions. In chicken proinsulin mRNA, a 32-base extension in the 5'-end sequence, possibly derived from fluctuation of the TSSs, was shown to play important biological roles due to harboring two so-called upstream ATGs that throttle the translation from the downstream authentic ATG. Further detailed manual and computational functional annotations of the PAP data obtained in the present study to assess gains and losses of protein motifs as well as the sequence motifs in the 5'UTRs would further en-

rich our knowledge about the functional diversification of the protein functions resulting from the alternative use of the first exons.

PAPs as an additional complex trait of the human gene transcriptome

In addition to the alterations in the transcriptional regulation and downstream coding sequences resulting from the use of APs in a single gene, the combinatory use of multiple APs between adjacent genes would enable further complex regulation of gene expression. Recent progress in genome research suggests that the transcriptome of human genes has greater than previously thought complexity (Kapranov et al. 2005), including transcripts generated from many so-called overlapping genes and anti-sense genes (Kiyosawa et al. 2003; Imanishi et al. 2004; Dahary et al. 2005). Newly identified additional PAPs (and thus first exons) have expanded the transcript regions (from TSSs to transcript termination sites), which had been delimited by RefSeqs, in many cases. Figure 6A shows the length distribution of the transcript regions extended towards the 5'-end by the present study. Occasionally, the extensions lead to the identification of novel cases in which adjacent gene pairs are overlapping with each other either in the same or opposite direction (210 and 360 gene pairs, respectively; Fig. 6B; typical examples are shown in Supplemental Fig. 4). In those cases, it is possible that the alternative first exons are also involved in the regulation of the adjacent genes by interfering with the transcription from the genomic regions that overlap with each other. Even among PAPs belonging to the same gene, it is possible that some mutual influences are exerted. In the yeast SER3 gene, transcription from the upstream region of the canonical promoter is indispensable for the proper transcriptional control of the gene, masking the regulatory DNA elements of the downstream promoters, and thereby repressing the transcription (Martens et al. 2004). Similarly, for some PAPs in human genes, transcription from the distal (upstream) promo-

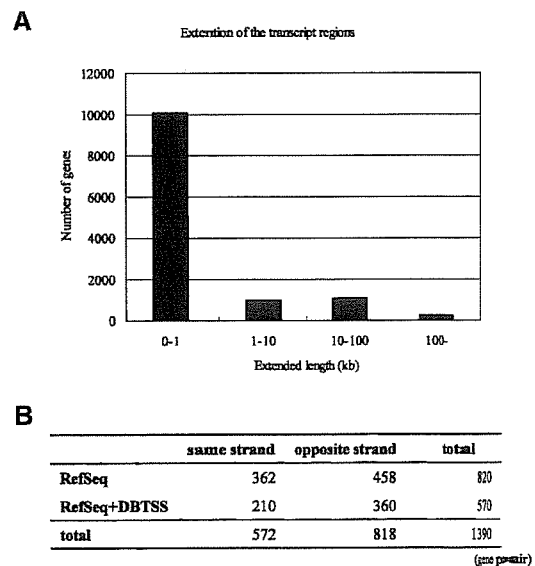


Figure 6. Identification of putative overlapping and anti-sense gene pairs. (A) Length distribution of the RefSeq (LocusLink) regions extended by the additional DBTSS data. (B) Number of putative gene pairs identified using the indicated data set.

ters might be occurring and might consequently inhibit transcription initiation from the proximal (downstream) promoters. Although it still largely remains unclear whether there is mutual dependence of the transcriptional regulatory effects between multiple promoter units, the data set of the PAPs (and overlapping/antisense genes) produced in the present study will serve as an important basis for further exploration of the complex world of the transcriptome.

Conclusions

Here we described the genome-wide identification and characterization of the PAPs in human genes. In this study, based on analyses of the unprecedented amount of our newly generated oligo-cap cDNA data, we demonstrated that an unexpectedly large population of human genes is subject to regulation by more than a single promoter. The diversity of the transcriptional modulation and the consequent alterations of the N termini of the encoded proteins revealed that the PAPs have the potential to play major roles in generating the complexity of human gene expression. Indeed, PAPs were shown to have at least as much potential as AS in this regard.

This is the first report that describes what is actually occurring in the immediately proximal regions around the sites of transcription initiation within the cell and shows that these events increase the functional complexity of the genes. Although there was a pioneering study of potential PAPs using EST data by Landry et al. (2003), the 5'-end completeness of each PAP, and thus whether it represented an AP or AS of an internal exon, was not clearly discriminated in their survey. Besides, our analysis of the first exon patterns (Fig. 4) indicated that our newly and massively generated 5'-end data are qualitatively different from the previously available dbEST data, and therefore precise analysis of the PAPs was previously essentially impossible.

For a number of other mammals, such as mouse, rat, dog, and various kinds of primates, full-length cDNA data together with the genomic sequences have now become available or will become available in the near future. Recently developed high-throughput TSS detection technologies (Shiraki et al. 2003; Hashimoto et al. 2004) will further accelerate the accumulation of positional information of the TSSs. Detailed comparative studies of each of the PAPs, for example, studies of which PAPs and which of their parts are conserved during evolution and which have been diversified, would provide us with key information about the putative ancestral forms of transcriptional modulation as well as information about how such modulation has evolved in each organismal lineage (Xie et al. 2005). Indeed, it has long been supposed that the genetic basis for various anatomical and physiological similarities/differences between humans and other organisms lies in alterations in the expression of genes rather than changes in the functions of their encoded protein products (King and Wilson 1975; Tautz 2000; Boguski 2002). To address these issues, various types of data regarding biological activities and evolutionary conservation of the promoters are actually being accumulated. In particular, it should be extremely fruitful to interpret the upcoming data from the ongoing ENCODE project (ENCODE Consortium 2004; <http://www.genome.gov/10005107>) in the context of the PAPs identified in the present study. This study gives a firm base for further exploring the molecular basis underlying the complexity of the transcriptional regulation of the human genome.

Methods

Mapping of the 5'-end sequences of the full-length cDNAs, filtration of the possibly dubious cDNAs, and classification of the putative TSSs into putative alternative promoter groups

We isolated 1,780,295 human cDNA sequences from full-length cDNA libraries constructed from 164 kinds of human tissues and cultured cells. The detailed library information is provided as Supplemental information. Each of the cDNA libraries was constructed using the oligo-capping method. Part of the sequence data was used for further selecting putative novel transcripts in the FLJ cDNA project (Ota et al. 2004). The computational mapping of the cDNA sequences onto the genomic sequence (hg_17; obtained from UCSC Genome Browser) was done by sequential use of BLAT and SIM4, following the procedure described in our previous reports (Suzuki et al. 2004). Positional information of the RefSeq genes is as of hg_17. Those cDNA sequences that could not be mapped from their first bases were excluded from the data set for the precise identification of the TSSs. Furthermore, to minimize the frequency of erroneously oligo-capped truncated cDNAs, the 5'-ends of the cDNAs that mapped inside of the second or later exons of any other cDNAs or RefSeq were excluded. As described in the text, this filtration was intended for removing the dubiously identified truncated cDNAs from the data set. We presumed that those cDNAs whose 5'-ends were located outside of the exonic regions of any other cDNAs could not be truncated forms of any known types of transcripts at least (for schematic representation, see Fig. 1, bottom line). We would like to add the caveat that the PPRs that were genuinely located inside the exonic regions of other transcript variants had been missed in exchange by this procedure. To rescue them, we also proposed an extensive procedure as shown in Supplemental Figure 5. Ten-base margins were also allowed for each of the exon-intron boundaries for the judgment. This was done to diminish the influence of inaccurate mapping. The cDNA sequences overlapping with any of the RefSeq sequences belonging to a given Locus ID were considered to correspond to that Locus ID (as of February 10, 2005; obtained from the NCBI download site). The locus-grouped sequences were further separated into several TSS clusters by binning the TSSs by 500-bp intervals. All of the raw data together with the obtained results are presented in our DBTSS data set (the Web site will be open to the public on acceptance of this manuscript).

Characterization of the promoter motifs

The presence of CpG islands was determined using NEWCPGREPORT contained in the EMBOSS package 2.8.0 (Rice et al. 2000). The CpG islands covering the TSSs were counted as "CpG island-containing" promoters. For the search of TATA boxes, MATCH was run for TRANSFAC database version 8.2 (Wingender 2004; <http://www.gene-regulation.com/>) using the matrices V\$TATA_01 and V\$TATA_C with the search conditions of: plus strand; -43 to -27; cut-off values of minFN74.pr. The obtained hits were counted as "TATA-containing" promoters.

Categorization of the first exons

For every combination of the first exons belonging to the same PAP group, the exon patterns were classified into the categories illustrated in Figure 4. Concerning the 3'-end boundaries of the first exons and the 5'-end boundaries of the second exons, only the patterns with a mutual difference >10 bp were counted. For counting the total number of "TSS variables," which is the total number of the 5'-end boundaries of the first exons (TSSs) in this population, such an allowance was not made since the precise

mappings were assured at the initial mapping process. Then, to identify the first exon patterns that were responsible for separating PAP groups, the patterns whose TSSs were separated by >500 bp were counted.

Calculation of the statistical significance

Statistical significance of the tissue-specific usage of the PAPs and enrichment of the GO terms was evaluated by calculating the hyper-geometric distribution using the following equation.

$$\sum_{x=i}^{Np} \frac{\binom{Np}{x} \cdot \binom{N(1-P)}{n-x}}{\binom{N}{n}}$$

where N = total number of cDNAs belonging to the locus, P = expected frequency of the cDNAs associated with a given tissue or a given GO term within the locus (number of associated cDNAs/ N), n = total number of cDNAs belonging to the PAP of concern, and i = number of the cDNAs associated with a given tissue or a given GO term within the AP.

For the tissue classification of the cDNA libraries, see Supplemental Table 1. GO terms were related to the LocusLink genes according to "loc2go" obtained from LocusLink.

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Generation of Green Fluorescent Protein-Expressing Monkey Embryonic Stem Cells

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Summary

Monkey embryonic stem (ES) cells are a useful tool for studying early human development and evaluating the efficacy of stem cell therapy. Monkey ES cells show closer similarity to human ES cells than their mouse counterparts regarding morphology, cell surface markers, and the maintenance of pluripotency, including the leukemia inhibitory factor requirement. The generation of genetically modified monkey ES cells with a biomarker such as green fluorescent protein, which allows noninvasive monitoring of progeny ES cells, is invaluable for the development of cell transplantation therapy and the study of differentiation mechanisms in primates. Here, we describe the generation of green fluorescent protein-expressing monkey ES cells using a conventional electroporation method.

Key Words: Electroporation; gene transfer; genetic modification; GFP; monkey ES cell.

1. Introduction

Establishment of human embryonic stem (ES) cells (1,2) and the development of nuclear transfer are the fundamental technologies for the clinical application of stem cell therapy in regenerative medicine. Generation of autologous ES cells by human therapeutic cloning through somatic cell nuclear transfer has been proposed for cell transplantation therapy (3). However, ethical concerns are the major obstacle to using human ES cells, especially to perform human therapeutic cloning.

To circumvent ethical issues and evaluate the efficacy and safety of cell transplantation therapy, an accurate animal model is required. Although mouse ES cells have been widely used for differentiation studies, there are significant differences between mouse and human early development and their ES cells (4).

Monkey ES cells, on the other hand, share many characteristics with human ES cells (4–8). For example, monkey ES cells show similar morphology and expression pattern of the cell surface markers, such as stage-specific embryonic antigens (SSEAs) (e.g., SSEA-3, SSEA-4) and tumor rejection antigens (TRAs) (e.g., TRA-1-60, TRA-1-81) to human ES cells. Maintenance of pluripotency and self-renewal of monkey and human ES cells largely depends on the unknown factors provided by mouse embryonic fibroblasts (MEFs), and unlike mouse ES cells, their pluripotency cannot be maintained with leukemia inhibitory factor (LIF) in the absence of MEFs. In addition, early development of nonhuman primate and humans is basically quite similar. Therefore, monkey ES cells are considered the ideal model for studying early human development and the development of stem cell therapy.

Genetic modification of ES cells by introducing disease-related genes and gene targeting is also key for the clinical application of stem cell therapy (9). However, monkey ES cells tend to differentiate when dissociated into single cells, which is required for most of transfection procedures. This results in inefficient transfection and has limited their application in biomedical research (10). We found that electroporation is a reliable method to introduce foreign genes into monkey ES cells without jeopardizing their pluripotency (11), although it is not as efficient as that of mouse ES cells.

In this chapter, we describe protocols to introduce the enhanced green fluorescent protein (EGFP) gene into monkey ES cells. The EGFP-expressing monkey ES cells provide a powerful tool to study pluripotency in ES cells by monitoring their differentiation and tissue regeneration processes. The capability to distinguish ES cells from the host cells should allow the accurate validation of clinical applications of cell transplantation therapy.

2. Materials

2.1. Tissue Culture

1. Phosphate-buffered saline (PBS): dissolve 9.6 g Dulbecco's PBS (–) powder (Nissui, Tokyo, Japan; cat. no. 05913) into 1 L distilled water (dH₂O) and autoclave.
2. 0.25% trypsin ethylenediaminetetraacetic acid solution (Nacalai, Kyoto, Japan; cat. no. 35554-64).
3. 0.1% collagenase solution: dissolve 0.1 g collagenase (Wako, Osaka, Japan; cat. no. 034-10533) in 100 mL Dulbecco's modified Eagle's medium (DMEM), filter, aliquot into 10-mL portions, and store at –20°C.
4. 0.1% gelatin: dissolve 0.5 g gelatin (Sigma, St. Louis, MO; cat. no. G1890) in 500 mL dH₂O and autoclave. Store at 4°C.
5. Mitomycin C (MMC; Nacalai, cat. no. 23305-94): dissolve 10 mg powder in 10 mL dH₂O, filter, and store at –20°C protected from light.
6. DMEM (Nacalai, cat. no. 14246-25).
7. DMEM/F12 Ham (Sigma, cat. no. D6421).
8. 100 mM sodium pyruvate solution (Sigma, cat. no. S8636).
9. Nonessential amino acids (NEAA) solution (Sigma, cat. no. M7145).
10. 200 mM L-glutamine solution (Nacalai, cat. no. 16948-04).
11. 2-Mercaptoethanol (Sigma, cat. no. M7522).
12. Knockout serum replacement (KSR; Invitrogen, Carlsbad, CA; cat. no. 10828-028).

13. Penicillin-streptomycin solution, 5000 U/mL penicillin, 5 mg/mL streptomycin (100X; Nacalai, cat. no. 26252-94).
14. Fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS; cat. no. 12103-500M).
15. Falcon cell strainer (100 μ m; BD Biosciences, Bedford, MA; cat. no. 352360).
16. Iwaki polystyrene 100-mm Petri dishes (Asahi Techno Glass, Tokyo, Japan; cat. no. 1020-100X).
17. Iwaki polystyrene 100-mm tissue culture dishes (Asahi Techno Glass, cat. no. 3020-100X).
18. Iwaki polystyrene 60-mm tissue culture dishes (Asahi Techno Glass, cat. no. 3010-060X).
19. Iwaki polystyrene 35-mm tissue culture dishes (Asahi Techno Glass, cat. no. 3000-035X).
20. Nunc four-well multidish (Nalge Nunc, Rochester, NY; cat. no. 176740).
21. MEF medium: MEF cells are maintained in DMEM with 10% heat-inactivated FBS, 2 mM L-glutamate, and 1% penicillin-streptomycin. To prepare a bottle of MEF medium, combine 57 mL FBS, 6 mL L-glutamate, 6 mL penicillin-streptomycin, and 500 mL DMEM. Store at 4°C.
22. ES medium: cynomolgus monkey ES cells (CMS-A2, CMK6) (7,11) are maintained in DMEM/F12 Ham containing 20% knockout serum replacement, 1% NEAA, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1% penicillin-streptomycin. To prepare a bottle of monkey ES medium, add 5 mL NEAA, 5 mL sodium pyruvate, 5 mL L-glutamine, 5 mL penicillin-streptomycin, 4 μ L 2-mercaptoethanol, and 100 mL knockout serum replacement into 400 mL DMEM/Ham F12. This medium is stable at least 3 wk when stored at 4°C.

2.2. Electroporation

1. QIAfilter Plasmid Midi kit (Qiagen, Valencia, CA; cat. no. 12243).
2. Alw44 I (ApaL I) (Toyobo, Osaka, Japan; cat. no. 031000).
3. Electroporation cuvet (Bio-Rad, Hercules, CA; cat. no. 165-2088).
4. Neomycin-resistant primary MEF cells (Specialty Media, Phillipsburg, NJ; cat. no. PMEF-N).
5. G-418 50-mg/mL solution (Nacalai, cat. no. 165-13).
6. ES cell characterization kit (Chemicon, Temecula, CA; cat. no. SCR 001).
7. Freezing vessel (Nihon Freezer, Tokyo, Japan; cat. no. BICELL).

3. Methods

3.1. Preparation of MEFs

Monkey ES cells require a mitotically inactivated MEF layer for the maintenance of pluripotency and self-renewal activity. The quality of MEFs critically affects the condition of monkey ES cells (*see Note 1*).

1. Sacrifice pregnant mice at 12.5 d postcoitum (dpc).
2. Remove the uterus to a 10-cm sterile Petri dish containing 10 mL PBS.
3. Dissect embryos from the uterus and place them in a new 10-cm sterile Petri dish containing 10 mL PBS.
4. Remove the upper part of the head and internal organs. Wash embryos with PBS in a new 10-cm dish to remove as much blood as possible.
5. Place embryos in a disposable 2.5-mL syringe attached to a 23-gage needle. Mince embryos by inserting plunger into the syringe and passing the embryos through the needle into a new 10-cm tissue culture dish containing 10 mL MEF medium (*see Note 2*). Aspirate partially minced embryos into the syringe and expel them again using the

plunger. Repeat this step until embryos are well disrupted into small pieces, then dispersed them in the medium uniformly. Incubate at 37°C, 5% CO₂, in a humidified atmosphere.

6. When cells reach confluence, trypsinize, filter tissue clumps out using a 100- μ m cell strainer, and freeze cells at a concentration of 5×10^6 cells/mL.

3.2. MMC Treatment of MEFs

1. Thaw a frozen vial of MEF cells in a 37°C water bath quickly, transfer into a 15-mL tube containing 5 mL MEF medium, and centrifuge (190g for 3 min). Aspirate medium, resuspend the cell pellet with fresh MEF medium, and plate in four new 10-cm dishes.
2. When confluent, adjust the medium to 5 mL and add 50 μ L MMC (10 μ g/mL final concentration).
3. Return dishes to the incubator for 2–3 h.
4. Wash the dishes three times with 10 mL PBS and trypsinize cells.
5. Either use MMC-treated cells immediately by plating $1\text{--}1.5 \times 10^6$ cells/6-cm dish or freeze for future use.

3.3. Culture of Monkey ES Cells

MEF feeder should be prepared 1–2 d before plating ES cells and used within 3–4 d of preparation (*see Note 3*).

1. Plate MEFs ($1\text{--}1.5 \times 10^6$ cells) in a gelatinized 6-cm dish the day before plating monkey ES cells.
2. Thaw a frozen vial of monkey ES cells (1×10^7) in a 37°C water bath quickly, transfer into a 15-mL tube containing 9 mL monkey ES medium, and centrifuge (190g for 3 min). Aspirate medium, gently resuspend the cell pellet with 5 mL fresh monkey ES medium, and plate into one 6-cm dish.
3. Change medium every day.
4. When the cultures reach confluence or if large colonies become predominant, then passage ES cells.

3.4. Passage of Monkey ES Cells

The timing of passage is important. Pass ES cells at about 50% confluence or before they form multilayer or donutlike (cells located in the center of the colony become flat and thin) colonies.

1. Remove medium and wash with 3 mL PBS.
2. Add 1 mL 0.1% collagenase to cover cell surface, then aspirate and return the dish to the incubator for 3–5 min (*see Note 4*).
3. Monitor ES colonies under the microscope. Undifferentiated ES colonies should shrink and separate from MEFs.
4. Add 2 mL ES medium and dissociate ES cells by gentle pipetting (*see Note 5*). The MEF layer also detaches from the dish by this procedure. Wash the MEF sheets by gentle pipetting to recover as much as ES colonies.
5. Transfer medium into a 15-mL tube; try to leave MEF clumps in the dish and centrifuge (190g, 3 min).
6. Aspirate medium and resuspend in 4 mL ES medium.

7. Seed the cells onto new feeder plates in the ratio of 1:2 to 1:4. Gently tilt the plates in both the *x* and *y* directions to distribute cells evenly. Then, incubate at 37°C. Cells will reach confluence in 3–4 d.

3.5. Vector Preparation

Use the GFP expression vector, which has EGFP gene under the control of the cytomegalovirus immediate-early enhancer and human elongation factor 1 α promoter (11,12) to express EGFP ubiquitously.

1. Purify supercoiled plasmid DNA with Qiagen column.
2. Linearize plasmid (100 μ g) using 100 U Alw44 I (ApaI) at 37°C overnight.
3. Confirm digestion by agarose gel electrophoresis.
4. Extract DNA with the same volume of phenol/chloroform/isoamylalcohol (25:24:1). After brief centrifugation (9000g, 2 min), transfer the aqueous phase to a new tube and precipitate DNA by adding 1/10 vol of 3 M sodium acetate (pH 5.2) and 2.2 vol of ethanol.
5. Recover DNA by centrifugation (18,000g, 15 min), wash with 70% ethanol, and dissolve in 10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (pH 8.0) at a concentration of 1 mg/mL. This DNA solution can be stored at -20°C.

3.6. Electroporation of Monkey ES Cells

The condition of ES cells affects transfection efficiency, especially survival after electroporation. Exponentially growing ES cells should be used.

1. Plate neomycin-resistant MEFs ($1-1.5 \times 10^6$) in a gelatinized 6-cm dish the day before electroporation of monkey ES cells.
2. Change ES cell media 2 h prior to electroporation.
3. Dissociate ES cells (approx 1×10^7) (see Note 6) with collagenase as described in Subheading 3.4.
4. Disperse ES cells into small clumps consisting of approx 5–10 cells by mechanical pipetting using a Pipetman (P-1000) (see Note 7).
5. Wash cells with ES medium by centrifugation (190g, 3 min). Aspirate supernatant and resuspend with 5 mL ES medium.
6. Transfer cell suspension to a gelatinized plate and incubate at 37°C for 10–20 min (see Note 8) to eliminate MEF feeders.
7. Collect floating monkey ES cell clumps by centrifugation (190g, 3 min), wash with 10 mL PBS (190g, 3 min), and resuspend in 0.8 mL PBS.
8. Mix the cell suspension with 20–40 μ g linearized plasmid DNA and transfer to an electroporation cuvet (4-mm gap).
9. Place the electroporation cuvet on ice for 10 min.
10. Set up the conditions for electroporation (500 μ F, 250 V, Gene Pulser II, Bio-Rad).
11. Transfer the cuvet into the cuvet holder of electroporator, then deliver electronic pulse (500 μ F, 250 V).
12. Remove the cuvet from the holder and incubate on ice for 10 min.
13. Resuspend cells with 8–12 mL of ES medium and plate into two or three 6-cm dishes with neomycin-resistant MEFs.
14. Change the media the next day.
15. At 48 h after electroporation, switch the medium to ES medium containing G418 (200 μ g/mL) for drug selection.

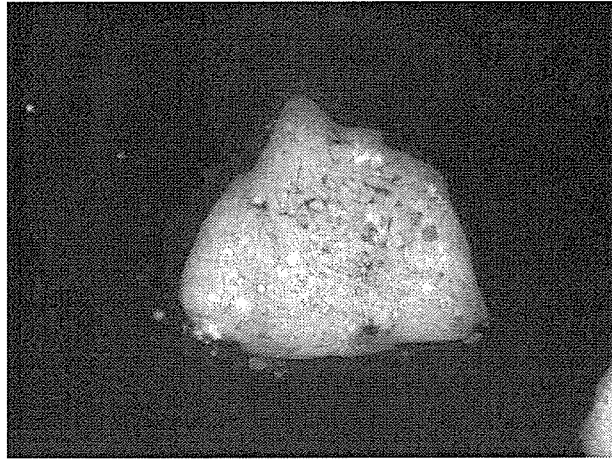


Fig. 1. Green fluorescent protein (GFP)-expressing monkey embryonic stem (ES) cell line. Fluorescent image of an undifferentiated monkey ES cell colony that stably expresses GFP. Strong and uniform GFP expression was observed. Individual cells and their shape were clearly identified by GFP expression.

16. Change the medium every day for 5–8 d. G418-resistant and GFP-expressing colonies will grow to a suitable size for pick up.

3.7. Isolation of GFP-Expressing ES Colonies

1. Plate neomycin-resistant MEFs (1×10^5) in a gelatinized four-well plate the day before picking monkey ES colonies.
2. Monitor GFP-expressing colonies under a fluorescent microscope. Mark the colonies to be picked on the bottom of the dish (*see Note 9*).
3. Place the plate on a dissecting microscope and pick up colonies using a flame-drawn Pasteur pipet.
4. Transfer the colonies into a drop (20–50 μ L) of ES medium prepared in a 3.5-cm dish and dissect the colony into several clumps using a 22–26-gage needle or flame-drawn glass capillary (*see Note 10*).
5. Transfer cell clumps into a four-well MEF plate containing 0.5 mL ES medium with G418.
6. When the culture reaches confluence, dissociate ES cells with collagenase and expand culture size to a 3.5-cm dish, then a 6-cm dish (*see Fig. 1*).
7. Confirm the expression of cell surface markers (alkaline phosphatase, SSEA-4, TRA-1-60, TRA-1-81) characteristic of monkey ES cells using an ES cell characterization kit according to the manufacturer's instructions.

3.8. Freezing of Transfected Cells

1. Collect confluent GFP-expressing ES cells from two 6-cm dishes by collagenase treatment followed by centrifugation (190g, 3 min).
2. Resuspend cells in 1 mL freezing medium consisting of 90% KSR and 10% dimethyl sulfoxide.

3. Transfer cell suspension into a cryotube and put it in a freezing vessel precooled at 4°C (*see Note 11*).
4. Put the freezing vessel in an -80°C freezer for 24 h.
5. Transfer cryotubes into liquid nitrogen.

4. Notes

1. It is advisable that a newly prepared MEF layer should be tested before use.
2. We usually use one or two embryos per 10-cm dish.
3. We routinely maintain monkey ES cells on MMC-treated MEF layer in 6-cm dishes. Culture and passage of monkey ES cells require some experience.
4. 0.25% trypsin containing 1 mM CaCl₂ and 20% KSR also works (7).
5. This step is very important and critical. Do not dissociate ES cell colonies into single cells as they differentiate or die. Check the dissociation under the microscope periodically. We usually dissociate into cell clumps consisting of approx 50 cells.
6. We routinely use three or four confluent 6-cm dishes or one or two 10-cm dishes for each electroporation experiment.
7. Try to minimize pipetting and bubble formation as it may damage ES cells.
8. Check periodically. Do not leave the plate in the incubator for a long time. Monkey ES cell colonies adhere easier to the plate than mouse ES cells do.
9. Try to pick up well-grown colonies with uniform GFP expression. Do not pick up colonies showing weak or mosaic GFP expression.
10. Dissected clumps consist of about 30–50 cells. If a colony is not big enough to dissect, then just transfer the colony into the new four-well plate and culture for 4–7 d more. When it grows to a suitable size, pick it up again and do mechanical dissection as described.
11. A Styrofoam tube stand for 15-mL culture tubes can be used as a freezing box.

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