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An exon-trapping system with a newly constructed trapping vector pEXT2; its application to the proximal region of the human chromosome 21 long arm

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We have developed an exon-trapping system with a newly constructed trapping vector containing multiple cloning sites (designated pEXT2). The system revealed high sensitivity for trapping a control exon from several hundred kbp of DNA. We have applied the system to the cosmid clones located on human chromosome 21p11–q21, and identified two fragments highly homologous to neurofibromatosis 1 (NF1) gene and a clearly transcribed fragment hybridized with ~1.6 kb RNA from human brain and human glioblastoma A172 cell.

Gene cloning; Plasmid vector; RNA splicing; Chromosome 21; Neurofibromatosis 1; Alzheimer's disease

1. INTRODUCTION

Recent advances in human genetic and physical mapping enable us to identify genes responsible for human disease by positional cloning [1]. In this strategy, once a location of the responsible gene is determined by genetic linkage analysis, it is possible to approach the gene by isolating candidate genes from corresponding genome segment. Several methods are available for the isolation of genes from specified genomic segments, which involve search for interspecies cross-hybridization (zoo blot) [2], sequence-based exon prediction [3], CpG islands [4], promoters [5] and enhancers [5, 6]. However, these methods cannot provide transcribed sequences directly.

An exon-trapping system has been recently developed, which directly provides possible exons by utilizing RNA splicing reaction carried out in mammalian cultured cells [7–10]. Selection of a trapping cassette in the exon-trapping vector is a crucial matter for the sensitivity and specificity of the system. Several genes were used as trapping cassettes in the previously reported system [7–10], whereas the efficiency of these trapping cassettes in their systems seemed to be variable.

Here, we report the realisation of our exon-trapping system using a newly constructed trapping vector, in which the rat α2 macroglobulin gene was used as a trapping cassette and multiple cloning sites were constructed (pEXT2). The system revealed high sensitivity for trapping a control exon from several hundred kbp of DNA. Using this system, we have identified two fragments highly homologous to the neurofibromatosis 1 (NF1) gene and a discretely transcribed fragment from the cosmid clones located on human chromosome 21p11–q21.

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Abbreviations. NF1, neurofibromatosis 1; RT, reversetranscription; PCR, polymerase chain reaction; APP, amyloid β protein precursor; SOD1, superoxide dimutase 1;

2. MATERIALS AND METHODS

2.1. *Vector construction*

The exon trapping vector pEXT2 was constructed as follows. A ~2.4 kbp of *Pvu*II fragment from the plasmid pUC13 was ligated with a *Bgl*II linker to yield pUC-*Bgl*II. The plasmid pSV2A/L-AΔ5′ [11] was cut with *Xba*I and self-ligated to yield pSV2A/ΔL. After removing the *Hin*dIII sites in pSV2A/ΔL by blunt-end cloning, the ~1.3 kbp of *Bam*HI fragment containing an SV40 early promoter, a remaining ~70 bp of the luciferase gene and a SV40 poly-adenylation signal, was cut from the plasmid and inserted into the *Bgl*II site of pUC-*Bgl*II (designated pUCSV2).

A 2.3 kbp of *Eco*RI–*Stu*I fragment containing exon 1, intron 1 and part of exon 2 of the rat α2 macroglobulin gene (position 4369– 6619) [12], was inserted into the *Eco*RI and filled-in *Bam*HI sites of pUC13. The remaining *Bam*HI site in the plasmid was removed by blunt-end cloning, and the *Bgl*II site (position 4541) [12] was also removed by insertion of an *Xba*I linker. The resultant *Xba*I fragment was cut from the plasmid and inserted into the *Xba*I site of pUCSV2

in the sense orientation to the SV40 early promoter (designated pEXT1). Finally, pEXT1 was cut with *Eco*RV and *Pst*I, and ligated with pre-annealed oligonucleotides, 5'-ATCAAGCTTGGATCCG-GAATTCACGGCCGCTGCA-3' and 5'-GCGGCCGTGAATTC-CGGATCCAAGCTTGAT-3', to yield pEXT2 with multiple cloning sites.

2.2. *Primers and PCR conditions*

The PCR primers used in the present study were as follows. F3, 5'-TTCCGGTACTGTTGGTAAAAT-3', R2, 5'-TCTGTAGGTA-GTTTGTCCAATTAT-3'; MacF, 5'-GCCTCAGCTCCACAAAAA-CC-3'; MacR, 5'-GAGGGAACCATCACCATGTAG-3'; T7-MacR, 5'- CGTAATACGACTCACTATAGGGAGGGAACCATCACCAT-GTAGA-3'. All reactions were performed with 200 nM of primers and 2.5 U of *Taq* DNA polymerase in *Taq* polymerase buffer consisting of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% Gelatin and 200 μM dNTPs. The thermal cycle conditions for PCR amplification were 94°C for 1 min, 55°C for 2 min, 72°C for 3 min.

2.3 *Selection of cosmid clones*

A cosmid library was constructed using Lorist2 [13] and the hamster–human hybrid cell line 2Fur which contains human chromosome 21 long arm as sole human component (21p11-qter) [14] The library was screened with human repetitive elements *Alu* and/or L1, resulting in the isolation of 33 clones. Each cosmid clone was then wholly labeled with [α-^{32}P]dCTP by random oligonucleotide priming, and used as a probe. Five micrograms of partially sheared genomic DNA from cultured cell lines, were alkali-denatured and dot-blotted onto a Hybond-N membrane (Amersham). Hybridization was carried out by the method of Corbo et al. [15], except that the pre-annealing time of DNA probes with human placental DNA was 2 h. The cell lines used for dot-blot analysis and their retained region of human chromosome 21 were as follows. WAV-17 (whole human chromosome 21 as sole human component)[16]; C2-T10 (21p12–qter)[17]; A2–4 (21pter–p12)[17]; 1881c-13b (21q22- qter)[18]. Human glioblastoma A172 and mouse fibroblast A9 were also used as controls. WAV-17, C2-T10, A2–4, A172 and A9 were purchased from ATCC, and 2Fur and1881c-13b were a gift from Dr. David Patterson

2.4. *Cell Culture, DNA transfection and RNA isolation*

COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 4.5% glucose and 100 μg/ml kanamycin. DNA transfection was performed by the method of Buckler et al. [8] with minor modifications. COS-7 cells grown to 80–95% confluency, were harvested and washed once with phosphate-buffered-saline containing no divalent cations (PBS(−)). The cells (4×10^6) were resuspended in cold PBS(−) (0.7 ml), put into a pre-cooled electroporation cuvette (Bio-Rad) and combined with plasmid DNA (10 μg in 0.1 ml of PBS(−)). After 10 min of incubation on ice, the cells were gently resuspended, electroporated with a Bio-Rad Gene Pulser (3 kV/cm; 25 μF; 200 Ω), and placed on ice for 10 min. The transformed cells were plated on a tissue culture dish (90 mm) containing 10 ml of prewarmed culture medium. After 48–72 h of transfection, the cells grown on the tissue culture dish were rinsed with PBS(−), and harvested by incubation with 10 ml of 0.05% (w/v) EDTA in PBS(−) at 37°C for 10 min Cytoplasmic RNA was isolated by a conventional method [19], and poly(A)$^+$ RNA was prepared by using a Magna PolyAAA$^+$ RNA Isolation Kit (Promega).

2.5 *Reverse-transcription (RT)-PCR*

Cytoplasmic poly(A)$^+$ RNA (0.3–0.6 μg) in the *Taq* DNA polymerase buffer was denatured at 65°C for 10 min. One mM of dNTPs, 5 μM of random hexanucleotides, 40 U of RNasin (Promega), and 200 U of SuperScript reverse transcriptase (BRL) were added to the reaction mixture, and then incubated at 42°C for 90 min (final vol. 20 μl). After the reaction, RNaseH (1 U) was added and incubated again at 37°C for 10 min. Half of the RT reaction was directly subjected to primary PCR amplification using the forward primer F3 and the reverse primer R2 for 30 cycles. The 0.3–0.9 kbp PCR products, size-fractionated by electrophoresis through a 1% agarose gel, were eluted and filtrated through a 0.45 μm membrane filter cartridge (UFC3–0HV, Miligen). The filtrate (1/1000th) was then subjected to secondary PCR amplification using the forward primer MacF and the reverse primer MacR for 35 cycles. The secondary PCR products were electrophoresed again through 3–3.5% agarose gel. The 50–600 bp fragments were purified and cloned into pBluescript II (Stratagene) by the T-Vector method [20].

2.6. *DNA sequencing*

Trapped fragments were sequenced on the ALF DNA sequencer with an AutoRead DNA sequencing kit (Pharmacia). The cycle sequencing was carried out with dsDNA Cycle Sequencing System (BRL)

2.7 *RNA blot hybridization*

Total RNA was extracted by the method of Chomoczynski and Sacchi [21], and poly(A)$^+$ RNA was isolated by Oligotex-dT30 (Roche). RNA was denatured by formaldehyde and electrophoresed through formaldehyde/1% agarose gel A Hybond-N nylon membrane (Amersham) was used for blotting. Trapped fragments cloned into pBluescript II were PCR amplified using the forward primer MacF and the reverse primer T7-MacR which the latter contains the T7 promoter sequence prior to the MacR primer [22], with the conditions described above except for the annealing temperature at 60°C. Riboprobes were synthesized from the PCR products using [α-^{32}P]UTP and T7 RNA polymerase Hybridization was carried out by the manufacture's recommended method (Stratagene) except for adding yeast tRNA (200 μg/ml) in the hybridization solution to suppress non-specific signals.

3. RESULTS

3.1. *Exon trapping system*

The final construct of the exon-trapping vector pEXT2 is shown in Fig. 1. The trapping cassette in pEXT2 consists of exon 1, intron 1 and part of exon 2 of the rat α2 macroglobulin gene, which is under the control of SV40 early promoter (Fig. 1A). The multiple

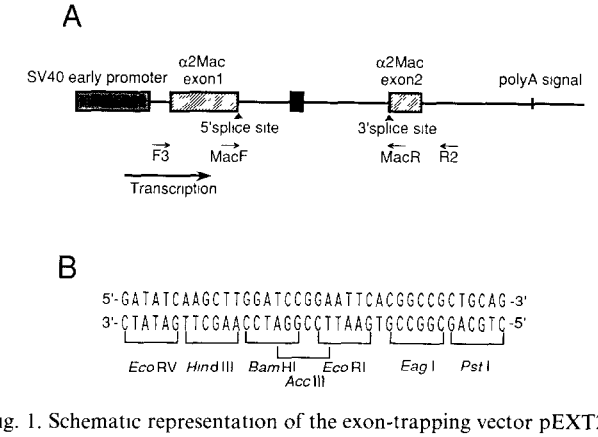

Fig. 1. Schematic representation of the exon-trapping vector pEXT2. (A) Structure of the trapping vector. The SV40 early promoter, and exon 1 and 2 of the rat α2 macroglobulin gene are described above each box. The horizontal line between exon 1 and 2 represents the intron sequence. A solid box in the intron means multiple cloning sites. Small arrows indicate primers and a large arrow indicates direction of the transcription. (B) Nucleotide sequence of multiple cloning sites. The sequence is described in the same orientation to (A). Seven restriction enzyme sites unique in the plasmid pEXT2, are shown below the sequence.

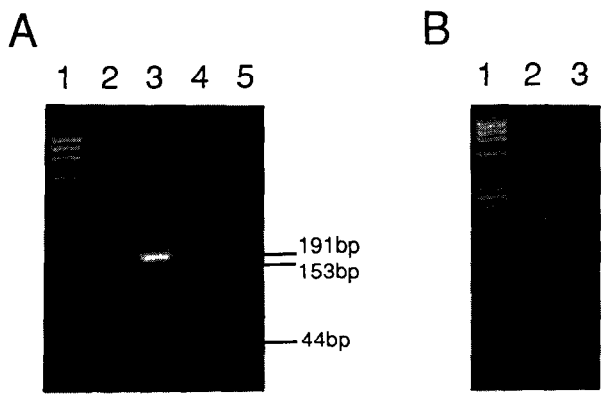

Fig. 2. Analysis of the RT-PCR products by agarose gel electrophoreses. (A) The secondary PCR products were electrophoresed through 3.5 % agarose gel. Lane 1 shows 200 ng of *Hae*III digested ϕ-X174 DNA marker. Lanes 2 to 5 show the PCR products derived from the plasmids described below. Lane 2, pEXT2 with no insert; lane 3, pEXT2/APPexon17S; lane 4, pEXT2/APPexon17AS, lane 5, plasmid mixture containing 150 independent clones and pEXT2/APPexon17S in the molar ratio 150:1 (see section 3). (B) An example of the secondery PCR products derived from the cosmid clone 379 (lane 2) and 478 (lane 3). Lane 1 shows 200 ng of *Hae*III digested ϕ-X174 DNA marker.

cloning sites consisting of seven unique restriction enzyme sites were constructed in intron 1 for inserting various DNA fragments (Fig. 1B). When pEXT2 is transfected into COS-7 cells, the trapping cassette is proposed to be transcribed and then spliced to produce a mature cytoplasmic poly(A)$^+$ RNA. If an appropriate fragment containing the entire exon and the flanking intron, is inserted into the cloning sites of pEXT2 in the sense orientation to the transcription, the exon is expected to be trapped between the exon 1 and 2 of trapping cassette in the mature cytoplasmic poly(A)$^+$ RNA. The trapped exon can be easily identified by reverse-transcription (RT)-PCR, since the RT-PCR product containing the trapped exon is longer than the non-trapped RT-PCR product.

To examine whether an appropriate exon is trapped ideally, a 1.2 kbp genomic fragment encompassing the exon 17 of amyloid β protein precursor (APP) gene [23] was inserted into pEXT2 in the sense and anti-sense orientations (designated pEXT2/APPexon17S and pEXT2/APPexon17AS, respectively). The plasmids were independently transfected into COS-7 cells, and cytoplasmic poly(A)$^+$ RNA, extracted from the cells after 2 days of incubation, was amplified by RT-PCR (see section 2). Fig. 2A shows an analysis of the secondary PCR products. The expected band of 191 bp was observed only when the genomic fragment was inserted in the sense orientation (lane 3). The corresponding band was sufficiently detected even after the primary PCR was carried out (data not shown). We sequenced the 191 bp band directly by cycle sequencing, and confirmed that the exon 17 of APP gene was precisely trapped between the exon 1 and 2 of the rat α2 macroglbulin gene (data not shown). On the other hand, a 44 bp band corresponding to non-trapped fragment was observed in the lanes of pEXT2 with no insert and pEXT2/APPexon17AS (lanes 2 and 4).

In the next step, we evaluated the sensitivity of the system. The 0.6–6 kbp of *Hin*dIII-digested inserted DNA, derived from the human chromosome 21 library (LL21N02, ATCC) [24], was inserted into the *Hin*dIII site of pEXT2. The plasmid DNA was prepared from 150 independent colonies and mixed with pEXT2/APPexon17S in the molar ratio 150 : 1. The mixed plasmid DNA was subjected to exon-trapping and the RT-PCR products were analyzed by agarose gel electrophoresis. Two trapped bands strongly stained were observed (Fig. 2A, lane 5). They were cloned into pBluescript II and analyzed by sequencing. We confirmed that the upper band comparable to the band of lane 3, was, as expected, for the exon 17 of APP gene. On the other hand, the lower band, unexpectedly trapped, was for exon 4 of superoxide dismutase 1 (SOD1) gene which is located on human chromosome 21q22.1 [25] (data not shown). The results indicate that both the control exon and the exon 4 of the SOD1 gene were successfully trapped from 150 independent clones with a mean 4 kbp inserted fragment, thus several hundred kbp of DNA can be assayed at one time.

3.2. *Application of pEXT2 exon-trapping system to human chromosome 21p11–q21*

We have applied the system to the proximal region of the human chromosome 21 long arm, because few genes have been identified within the region [27], whereas some early-onset familiar Alzheimer's disease has shown a linkage to chromosome 21 DNA markers located on the region [26]. Thirty-three human chromosome 21 cosmid clones, obtained from a cosmid library constructed from the hamster–human hybrid cell line 2Fur which contains the human chromosome 21 long arm as sole human component (21p11–qter)[14], were screened by dot-blot analysis using the genome DNA from several rodent–human hybrid cell lines (see section 2). Twelve cosmid clones exhibited positive signals in WAV-17 and C2-T10 and negative signals in 1881c-13b, and therefore their locations were determined on 21p11–q21 (data not shown). The selected clones were subjected to exon-trapping independently. The *Sau*3AI partially cut DNA fragments from each cosmid clone were inserted into the *Bam*HI site of pEXT2. Plasmid DNA were prepared and transfected into COS-7 cells. The RT-PCR products were analyzed by agarose gel electrophoreses (Fig. 2B). The discrete bands were obtained from 6 of 12 cosmid clones and cloned into pBluescript II.

3.3. *Analyses of the trapped fragments*

Firstly we investigated the length and the existence of possible reading frames by sequencing all of the cloned

Table I

Characterization of trapped fragments. Trapped fragments were cloned into pBluescript II. The name of each clone was determined by a combination of the original cosmid number and the following sub-number. N.D., not determined.

Clone Name	Trapped Fragment Size (bp)	Reading Frame	DNA Sequence Homology Search	Northern Analysis
244-20	55	+		–
379-8	106	+		–
379-10	155	+		–
379-11	138	+		+ (1.6kb)
478-9	125	+		–
478-26	174	+	NF1 homologue	N.D.
661-19	41	+		–
1090-3	138	–		N.D.
1090-25	80	+	NF1 homologue	N.D
1113-13	200	+		–
1113-16	95	+		–

trapped fragments corresponding to the discrete bands (Table I). The length of eleven trapped fragments were 41–200 bp and mean 119 bp. Ten of eleven trapped fragments had possible reading frames. A computer search for sequence homology in Genbank and EMBL data library by using the FASTA program [28] revealed that clone 478–26 and 1090–25, which were obtained from independent cosmid clones, had almost the same nucleotide sequences to the 5′-terminal side of neurofibromatosis 1 (NF1) gene (Fig. 3). In spite of several nucleotide differences, these two clones still had a reading frame without frameshifts. The other trapped fragments had no significant homology to the nucleotide sequences of the database.

To investigate whether the trapped fragments with reading frames are expressed or not, RNA blot hybridization was performed. The trapped fragments on pBluescript II were PCR-amplified with a set of primers, MacF and T7-MacR, of which the latter contains the promoter sequence for T7 RNA polymerase prior to the MacR sequence. Riboprobes were synthesized from the PCR-amplified products and hybridized with poly(A)$^+$ RNA derived from human brain and human glioblastoma A172. Clone 379–11 showed a discrete hybridization signal of ~1.6 kb in both RNAs (Fig. 4).

4. DISCUSSION

In the present study, we firstly showed the realisation of an exon-trapping system with the newly constructed trapping vector pEXT2. We have selected the rat $\alpha 2$ macroglobulin gene as a trapping cassette, because the consensus sequences for RNA splicing, which involve 5′- and 3′-splice sites, a branch site and a pyrimidine tract, are sufficiently conserved in intron 1 of the gene. Furthermore, we have constructed multiple cloning sites in the intron to be able to conveniently insert DNA fragments cut with several restriction enzymes, whereas most of the trapping vectors reported previously had only one cloning site [8–10].

Using this vector, we showed that both exon 17 of the APP gene and exon 4 of the SOD1 gene were successfully isolated from the plasmid mixture, and that thus several hundred kbp of DNA can be assayed at a time. However, when the PCR product obtained from the plasmid mixture was applied to an acrylamide gel, we also detected several weak additional bands. Sequence analysis for such weak bands showed that the bands were unexpectedly spliced products using a cryptic splice site ~20 bp upstream from the multiple cloning sites (data not shown). Nonetheless, they did not seem very effective in our trapping system, since the unexpected cryptic splicing products represented only a very small portion of the PCR products. This may be explained by the fact that RNA processing using mutated splice sites comparable to the cryptic site is less efficient than that of normal splice sites [29]. Construction and evaluation of the exon-trapping vector from which such cryptic site is removed by site-directed mutagenesis, are in progress to further improve the system.

We have applied the system to human chromosome 21p11–q21. This region is estimated to be seven meg-

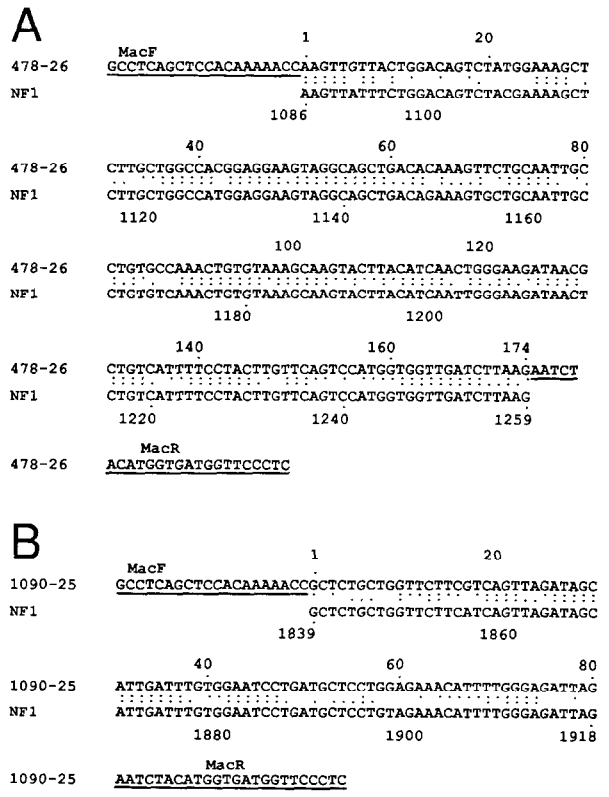

Fig. 3. Sequence comparisons between two trapped fragments and the neurofibromatosis 1 (NF1) gene. Two alignments between clone 478–26 and the NF1 gene (A), and between clone 1090–25 and the NF1 gene (B) are shown. Sequences from the trapping cassette are indicated by underlines. cDNA sequence of the NF1 gene and its nucleotide number are from Marcheuk et al. [40].

Fig. 4. Northen blot analysis of clone 379–11. A riboprobe synthesized from clone 379–11 were used for the hybridization. Lane 1, human glioblastoma A172 total RNA; lane 2, A172 poly(A)$^+$ RNA; lane 3, human brain poly(A)$^+$ RNA. RNA used in each lane was 2 μg. Autoradiography was performed at −70°C for 48 h with an intensifying screen.

abase pair long by the contig map which was constructed from yeast artificial chromosome clones covering the entire human chromosome 21q [30]. It is highly conceivable that this region may not be gene-rich, since the distribution of known transcribed sequences on the chromosome 21 long arm are not uniform and few genes were identified within the proximal region of this chromosome [27]. Therefore, this region seems to be suitable for the approach by exon-trapping, the efficient method to identify transcripts within a large genomic region. Furthermore, genetic linkage studies have revealed that some families of early-onset Alzheimer's disease co-segregated with chromosome 21 DNA markers located in this region [26].

Sequence analysis revealed two trapped fragments highly homologous to the NF1 gene. The NF1 gene is located on human chromosome 17 [31, 32], and until now, two NF1-related loci have been identified on chromosome 14 and 15 [33]. Therefore, the NF1-related locus identified on chromosome 21 in the present study may represent a pseudogene or a novel subtype of the NF1 gene. In any case, genomic analysis around the two trapped fragments should be necessary, since each of them still possesses a reading frame in spite of several nucleotide differences from the NF1 gene.

RNA blot hybridization showed that the trapped fragment 379–11 was transcribed. Two transcribed clones, M21 [34] and JG90 [35], are involved in the region investigated in the present study [27]. The hybridization signal of ~1.6 kb from clone 379–11 seems to be smaller than the cDNA size of M21 or the transcription size of JG90 (1.8 kbp or 3.5 kb, respectively) [34, 35]. Since clone 379–11 also has no homology to any known sequences in the database, the clone is considered to reflect an unknown gene. Its entire cDNA should be isolated for further characterization.

Eight of eleven trapped fragments did not show any sequence homology to known sequences nor discrete expression in both brain and glioblastoma cell A172, although most of them possess reading frames and therefore were candidate exons. One of possible explanations is that corresponding unknown genes are not expressed in brain but in other tissues, and/or are temporarily expressed only at certain stages of development. Further analysis of such trapped fragments may be necessary to ascertain whether they reflect expressed genes or not.

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