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Isolation of Human *purH* Gene Expressed in the Rodent Transformant Cells by Subtractive Enrichment of 3′-untranslated Region of Human Transcript

Masatake YAMAUCHI,[1,*] Naohiko SEKI,[2] Kazuei MITA,[1] Toshiyuki SAITO,[1] Satsuki TSUJI,[1] Etsuko HONGO,[1] Mitsuoki MORIMYO,[1] Tadahiro SHIOMI,[1] Hideki KOYAMA,[3] Dai AYUSAWA,[4] and Tada-aki HORI[1]

Genome Research Group, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263, Japan,[1] Laboratory of Gene Structure I, Kazusa DNA Research Institute, 1532-3 Yanauchino, Kisarazu, Chiba 292, Japan,[2] Division of Biochemistry, Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka, Totsuka-ku, Yokohama 244, Japan[3] Division of Molecular Cell Genetics, Kihara Institute for Biological Research, Yokohama City University, 641-12 Maioka, Totsuka-ku, Yokohama 244, Japan[4]

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Abstract

A subtraction procedure was developed for identification and isolation of a human gene transcribed in mouse transformant cells. The procedure was based on subtractive enrichment of the products that were amplified by the combination of reverse transcription and polymerase chain reaction from the 3′-untranslated region (3′-UTR) of human poly(A)+ RNA expressed in the mouse transformant cells. To assess the ability and usefulness of the procedure, we attempted to recover the human *purH* gene from a mouse transformant cell line, which was originally established by functional complementation using the human metaphase chromosome-mediated gene transfer technique from a mouse *purH*-negative mutant cell line. Using our procedure, a part of the human transcript in the transformant cells was successfully identified and isolated. The full-length cDNA was isolated using the 3′-UTR clone as a probe, and its biological activity was confirmed by introducing it into the mouse *purH*-negative mutant cells.

Key words: adenine auxotrophic mutant; *purH* gene; subtraction; 3′-untranslated region; RT-PCR

1. Introduction

Isolation of a recessive mutant from mammalian cultured cells and subsequent identification of its responsible gene has been one of the most successful approaches to investigate the biological function of a gene.

We have previously established the selection procedure for mouse recessive mutant cells.[1] This procedure was based on the synchronization of the cell cycle to the G1/S phase by treatment with aphidicolin, and subsequent enrichment of deficient mutant cells by the advanced [^{3}H]thymidine suicide technique using the mouse thymidylate synthase-negative mutant cell line[2] as a wild-type. Using the enriched cell population, mouse adenine auxotrophic mutants were isolated at the frequency of one in 2000 cells by replica plating.[1] Biochemical analyses of the three independent mutant cell lines (ade1, ade2, and ade3) revealed that they commonly lacked *purH* activities, completely or partially: Those are of 5-aminoimidazole-4-carboxamide -1-β-D-ribonucleotide transformylase (AICAR-TFase) and inosinicase, and correspond to *purH* in *Escherichia coli* (EMBL/GenBank Data Bases Acc. No. X51950, J02827, M32279), and Ade-F in Chinese hamsters.[3]

We have also established the chromosome-mediated gene transfer (CMGT) technique to introduce a human genomic gene at a higher frequency compared to the DNA-mediated one[4] by functional complementation of a recessive phenotype. This technique was successfully applied to the mouse adenine auxotrophic mutant cell lines and the Chinese hamster CTP synthetase (CTPS)-deficient mutant cell line.[5] The human CTPS gene was eventually cloned from a transformant cell line isolated using the CMGT procedure[6] for the analysis of the feedback mutations of the gene.[7]

However, conventional procedure to recover a human gene from rodent transformant cells was a labor-intensive and time-consuming process.[6,7,9,10] In a series of analyses of the CMGT transformant cell lines, the functional human genomic DNA fragments carrying the CTPS gene

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* To whom correspondence should be addressed. Tel. +81-43-251-2111, Fax. +81-43-251-9818

that was integrated into the Chinese hamster genome was found out to be unexpectedly smaller than reported by other research groups.[11,12] The majority of human DNA fragments carrying the functional CTPS gene that was integrated into the recipient Chinese hamster genome were not larger than 100 kb (6, 13, and our unpublished observations). This partly explained the low frequency of genomic gene transfer experiments, when the size of the genomic gene was relatively large. This, however, also suggested that it is unlikely that another functional human genomic gene would be transferred with the target gene simultaneously into the rodent recipient cells, although a large amount of human genomic DNA was detected in the CMGT transformant cells by Southern blot hybridization. These experiences led us to the idea to establish a new procedure that can identify and clone a human gene expressed in rodent transformant cells using a subtractive selection of poly(A)+ transcripts.

Our procedure is based on the subtractive selection of the 3′-untranslated region (3′-UTR) of poly(A)+ RNA. Since the 3′-UTR is relatively AT-rich and also is not highly conserved among different species compared to the coding region, hybridization under stringent conditions should be able to distinguish human transcript expressed in a transformant cell line from background rodent transcripts. The relatively short size of the 3′-UTR is also thought to be advantageous for poly(A)+ RNA preparation and subsequent RT-PCR.

In this study, we demonstrate the usefulness of our procedure in recovery of the human *purH* gene transferred to the mouse *purH*-negative mutant cells.

2. Materials and Methods

2.1. Cell lines

Mouse adenine auxotrophic mutant cell lines (ade1, ade2, and ade3) were isolated from the thymidine auxotrophic mutant cell line, FSthy-21,[2] of the mouse mammary tumor cell line, FM3A, by the advanced [^{3}H]thymidine suicide method of the synchronized cell culture and following replica plating.[1] The transformant cell line, S3161, was isolated by functional complementation of the adenine auxotroph of the mutant cells using a human (HeLa) metaphase chromosome-mediated gene transfer technique.[1] FSthy-21 cells and S3161 cells were maintained in ES medium (modified MEM, Nissui, Japan) supplemented with 2% fetal bovine serum (FBS) and 2×10^{-5} M thymidine. For adenine auxotrophic mutant cell lines, 10^{-4} M adenine was also added to the medium. HeLa cells were maintained in ES medium supplemented with 10% FBS.

2.2. Reverse transcription and polymerase chain reaction (RT-PCR)

Poly(A)+ RNA was extracted as previously described[14] and annealed to primer 1 in 20 μl of the reaction mixture containing 1 μg of poly(A)+ RNA, 10 mM PIPES buffer (pH 6.4), 500 mM KCl, 50 ng of primer 1, and10 units of RNAsin (Promega, USA), by heating at 85°C for 10 minutes and cooling down to 55°C. Reverse transcription was performed in 100 μl of the reaction mixture containing 20 μl of the annealing mixture, 50 mM Tris-HCl (pH 8.3), 2 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EDTA, 20 units of RNAsin, 200 μM dNTPs, and 10 units of AMV reverse transcriptase (Life Sciences, USA) at 42°C for 60 minutes. The products were precipitated by ethanol, vacuum-dried, and dissolved in 62 μl of distilled water. Second strand DNA synthesis and the following 30 cycles of PCR were performed in 100 μl of the reaction mixture containing 600 ng each of primers 2 and 3, and 5 units of Taq DNA polymerase (Biotech, Australia) by heat denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minutes. For PCR of the samples of the adenine auxotrophic mutants, 20 μM biotin-dUTP was also included in the reaction mixture. Nucleotide sequences of oligonucleotide primers were as follows: primer 1,5′-CTG AAT TCA CCC GGG ATA TTT TTT TTT TTT TTT TT-3′; primer 2,5′-CGA ATT CAA ATA AA-3′; primer 3,5′-CTG AAT TCA CCC GGG ATA-3′.

2.3. Subtractive enrichment

The RT-PCR product was de-proteinized by phenol extraction, precipitated with ethanol, and dissolved in distilled water. The product was then subjected to endonuclease digestion with *Eco*RI. For the samples of the adenine auxotrophic mutants, the digests were further treated with bacterial alkaline phosphatase (Takara, Japan). The digests were deproteinized by phenol extraction, precipitated with ethanol, dissolved in 90 μl of TE buffer, and 10 μl of 5 M NaCl and 1 μl of 20% SDS were added. 10 μl of DNA from transformant cells was mixed with 100 μl of DNA from recipient cells, boiled for 5 minutes, and incubated at 65°C for 48 hours for annealing. To the annealing mixture, 40 μl of streptavidin-agarose resin (SAR, 20 μl of packed resin, Life Technologies Inc., USA) was added, and incubated at room temperature for 30 min with continuous mixing. SAR was removed by centrifugation as recommended by the manufacturer. SAR treatment was repeated twice. The resin was removed completely through a Takara filter column to avoid the contamination of the biotinylated DNA. After phenol extraction, DNA was precipitated with ethanol, and resuspended in 10 μl of TE. The overall subtractive process was repeated twice. The resultant DNA was subcloned into a dephosphorylated pUC19 plasmid vector.

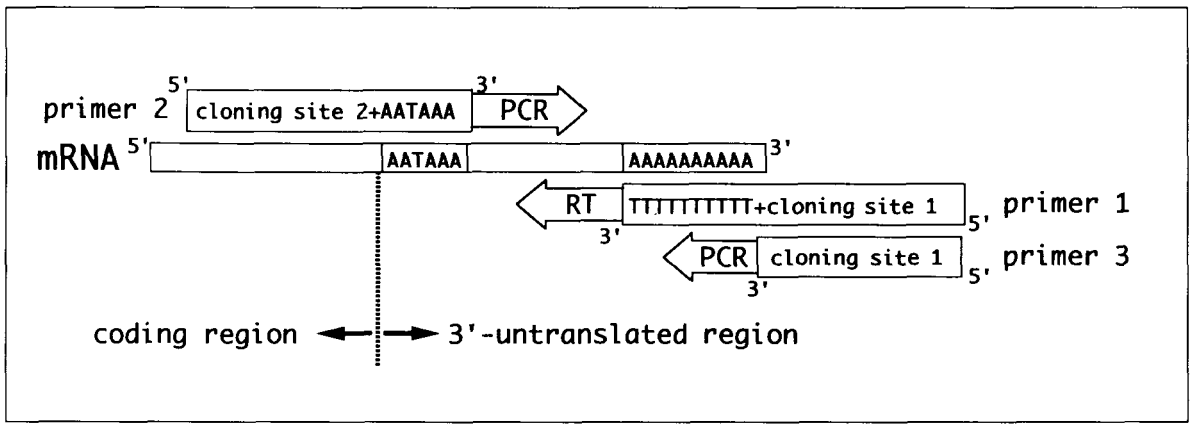

Figure 1. RT-PCR of 3′-untranslated region (3′-UTR) of poly(A)+ RNA. Poly(A)+ RNA was prepared from the mouse transformant cells, S3161, and reverse-transcribed (RT) using primer 1. The product was then amplified by polymerase chain reaction (PCR) using primer 2 and primer 3. For RT-PCR of the mouse recipient cells, ade 3, biotin-11-dUTP was also included in the reaction mixture as described in Materials and Methods.

Inserts of white colonies were amplified by polymerase chain reaction using M4 (5′-GTT TTC CCA GTC ACG AC-3′) and RV (5′-CAG GAA ACA GCT ATG AC-3′) universal primers, as described above.

2.4. Southern blot hybridization of subtracted samples

Colony PCR products were separated on 1.2% agarose gel and transferred to a nylon membrane. Hybridization was performed in 3xSSC-0.1% SDS-50% formamide system using the radio labelled total human genomic DNA extracted from HeLa cells as a probe under suppression conditions using Cot-I DNA (GIBCO-BRL, USA), oligo(dT)$_{20}$, and oligo(dA)$_{20}$. After hybridization, the membrane filter was washed in 0.1xSSC-0.1% SDS at 65°C twice and exposed to X-ray film.

2.5. Isolation of full-length cDNA

A full-length cDNA clone was isolated by plaque hybridization technique[6] using a human testis cDNA library (Clontech Laboratories Inc., USA).

2.6. DNA sequencing

Nucleotide sequence of the cDNA clones were determined by the cycle sequencing method automatically using ABI 373A DNA sequencer (Applied Biosystems Inc., USA), and confirmed by the dideoxy termination method[6] manually.

2.7. Complementation of mouse purH *mutation*

A full-length cDNA clone was subcloned into a mammalian expression vector pSV2E, a derivative of pSV2neo.[15] Plasmid DNA containing human *purH* cDNA was introduced into mouse *purH* mutant cell lines as calcium-phosphate co-precipitate as previously described.[1,6] After 1 week of cell culture under selective conditions without adenine, the number of colonies formed was counted and the transformation activity was calculated against the number of colonies on the control plates supplemented with adenine.

3. Results and Discussion

3.1. Subtractive enrichment of human mRNA

S3161 cells, the mouse transformant cells, were used for isolation of the human gene, since the least amount of the human genomic DNA was detected by Southern blot analysis.[1]

3′-UTR of poly(A)+ RNA was prepared by the RT-PCR technique using three oligonucleotide primers, oligo(dT)$_{17}$ with multicloning site (primer 1), poly(A) signal (primer 2), and the multicloning site of primer 1 (primer 3), as shown in Fig. 1. For PCR of the mouse recipient (ade 3) sample, biotin-dUTP was included in the reaction.

After *Eco*RI digestion and heat-denaturation, the RT-PCR product of the mouse transformant cells was mixed with 10 times excess amount of the product of the mouse recipient cells that was digested and dephosphorylated prior to heat-denaturation. The mixture was incubated at 65°C for 48 hours for annealing, and treated with streptavidin-agarose resin. During this process, the RT-PCR products of mouse transcripts from the transformant cells were expected to form duplexes with an excess amount of the biotinylated products from the mouse recipient cells, and removed by adsorption on the resin. Streptavidin adsorption was repeated twice to reduce the contamination of mouse transcript. The resultant product was precipitated with ethanol, and the steps of heat-denaturation, annealing, and streptavidin adsorp-

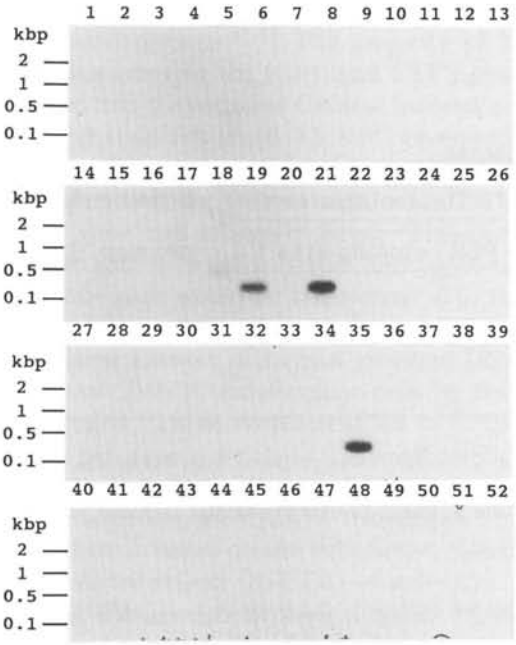

Figure 2. Detection of human-specific RT-PCR products recovered by 3′-UTR subtraction. After subtractive enrichment, the RT-PCR product was subcloned into pUC19 plasmid vector. White colonies on LB plates containing ampicillin and X-Gal were isolated, and the inserts were amplified by the colony PCR technique. The PCR product was subjected to agarose gel electrophoresis and blotted onto a nylon membrane. Hybridization was performed by using total human genomic DNA as a probe under suppression condition using Cot-I DNA, poly(dA)$_{20}$, and poly(dT)$_{20}$.

Table 1. Percent identify of the putative amino acid sequences of the *purH* genes. The putative amino acid sequence of the human *purH* gene given in the Fig. 3 was compared to those of *E. coli*, *B. subtilis*, and *chicken*.

	Human	Chicken	*E. coli*	*B. subtilis*
Human	100	83	34	32
Chicken		100	32	33
E. coli			100	51
B. subtilis				100

tion were repeated for further enrichment of the human products.

The final subtracted product was subcloned into the pUC19 plasmid vector that was digested and dephosphorylated.

3.2. Identification of human transcript

The ligated plasmid was introduced into DH5α, LacZ-negative *E. coli*, and white colonies formed on LB plates containing ampicillin and X-Gal were isolated and suspended in 100 μl of LB medium. One microliter of the suspension was amplified by PCR using universal primers in 10 μl of the reaction mixture as described in Materials and Methods. The product was subjected to 1% agarose gel electrophoresis and transferred to a nylon membrane. The membrane was hybridized with radiolabelled human probe, under suppression condition with human Cot-1 DNA, oligo(dT)$_{20}$ and oligo(dA)$_{20}$.

As shown in Fig. 2, three clones (#19, #21, and #35) out of 52 colony-PCR products gave significantly strong hybridization signals. Nucleotide sequence analysis of the three positive clones revealed that they were identical ones, although they did not show significant homology to the nucleotide sequences deposited in the EMBL/Genbank DataBase.

3.3. Isolation and characterization of full-length cDNA

One of the positive clones, clone #21, was used as a probe for screening full-length cDNA clones. The human testis cDNA library was screened by a plaque hybridization technique, and several clones were isolated.

Nucleotide sequence of the cDNA clone with the longest insert was determined (DDBJ/EMBL/GenBank nucleotide sequence data bases: accession number: D82348). As shown in Fig. 3, a single open reading frame was identified, and the nucleotide sequence of the 3′-UTR clone is underlined. Analysis of the putative amino acid sequence revealed that the human sequence was highly homologous to those from various species including *E. coli*, *B. subtilis*, and chicken. The result of amino acid sequence comparison is given in Table 1. The degree of similarity of the human sequence to those of chicken, *E. coli*, and *B. subtilis* was 83%, 37%, and 35%, respectively. This strongly suggested the cDNA clone isolated was human *purH*.

To analyse the biological function of the cDNA clone, the cDNA moiety of the clone was recloned into the mammalian expression vector (pSV2-HSpurH) and introduced into the mouse *purH*-negative mutant cell lines. As shown in Table 2, the cDNA clone with the insert in the right direction showed the activity in complementing the adenine auxotrophic phenotypes of three independent mutants at frequencies of about 10^{-5}–10^{-4}. In contrast, the plasmid with the cDNA insert in the opposite orientation and the plasmid with no insert did not form adenine prototroph colonies. Thus, we concluded that the cDNA clone isolated here was the human *purH* gene.

We described here the usefulness of our subtraction procedure in identification and isolation of a human gene expressed in mouse transformant cells. Our procedure is based on the fact that the nucleotide sequences in the 3′-UTR of poly(A)+ RNA from a housekeeping gene and its homologues vary among species, whereas the sequences in the coding region have been conserved. The human *purH* gene was successfully recovered by applying the

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cggcagccctcctacctgcgcacgtggtgccgctgctgctgcctcccgctcgccctgaacccagtgcctgcagccatggctcccggccag      90
                                                                                M  A  P  G  Q       5

ctcgccttatttagtgtctctgacaaaaccggccttgtggaatttgcaagaaacctgaccgctcttggtttgaatctggtcgcttccgga     180
L  A  L  F  S  V  S  D  K  T  G  L  V  E  F  A  R  N  L  T  A  L  G  L  N  L  V  A  S  G      35

gggactgcaaaagctctcagggatgctggtctggcagtcagagatgtctctgagttgacgggatttcctgaaatgttgggggacgtgtg     270
G  T  A  K  A  L  R  D  A  G  L  A  V  R  D  V  S  E  L  T  G  F  P  E  M  L  G  G  R  V      65

aaaactttgcatcctgcagtccatgctggaatcctagctcgtaatattccagaagataatgctgacatggccagacttgatttcaatctt     360
K  T  L  H  P  A  V  H  A  G  I  L  A  R  N  I  P  E  D  N  A  D  M  A  R  L  D  F  N  L      95

ataagagttgttgcctgcaatctctatccctttgtaaagacagtggcttctccaggtgtaactgttgaggaggctgtggagcaaattgac     450
I  R  V  V  A  C  N  L  Y  P  F  V  K  T  V  A  S  P  G  V  T  V  E  E  A  V  E  Q  I  D     125

attggtggagtaaccttactgagagctgcagccaaaaaccacgctcgagtgacagtggtgtgtgaaccagaggactatgtggtggtgtcc     540
I  G  G  V  T  L  L  R  A  A  A  K  N  H  A  R  V  T  V  V  C  E  P  E  D  Y  V  V  V  S     155

acggagatgcagagctccgagagtaaggacacctccttggagactagacgccagttagccttgaaggcattcactcatacggcacaatat     630
T  E  M  Q  S  S  E  S  K  D  T  S  L  E  T  R  R  Q  L  A  L  K  A  F  T  H  T  A  Q  Y     185

gatgaagcaatttcagattatttcaggaaacagtacagcaaaggcgtatctcagatgcccttgagatatggaatgaacccacatcagacc     720
D  E  A  I  S  D  Y  F  R  K  Q  Y  S  K  G  V  S  Q  M  P  L  R  Y  G  M  N  P  H  Q  T     215

cctgcccagctgtacacactgcagcccaagcttcccatcacagttctaaatggagcccctggatttataaacttgtgcgatgctttgaac     810
P  A  Q  L  Y  T  L  Q  P  K  L  P  I  T  V  L  N  G  A  P  G  F  I  N  L  C  D  A  L  N     245

gcctggcagctggtgaaggaactcaaggaggctttaggtattccagccgctgcctctttcaaacatgtcagcccagcaggtgctgctgtt     900
A  W  Q  L  V  K  E  L  K  E  A  L  G  I  P  A  A  A  S  F  K  H  V  S  P  A  G  A  A  V     275

ggaattccactcagtgaagatgaggccaaagtctgcatggtttatgatctctataaaaccctcacacccatctcagcggcatatgcaaga     990
G  I  P  L  S  E  D  E  A  K  V  C  M  V  Y  D  L  Y  K  T  L  T  P  I  S  A  A  Y  A  R     305

gcaagaggggctgataggatgtcttcatttggtgattttgttgcattgtccgatgtttgtgatgtaccaactgcaaaaattatttccaga    1080
A  R  G  A  D  R  M  S  S  F  G  D  F  V  A  L  S  D  V  C  D  V  P  T  A  K  I  I  S  R     335

gaagtatctgatggtataattgccccaggatatgaagaagaagccttgacaatactttccaaaaagaaaaatggaaactattgtgtcctt    1170
E  V  S  D  G  I  I  A  P  G  Y  E  E  E  A  L  T  I  L  S  K  K  K  N  G  N  Y  C  V  L     365

cagatggaccaatcttacaaaccagatgaaaatgaagttcgaactctctttggtcttcatttaagccagaagagaaataatggtgtcgtc    1260
Q  M  D  Q  S  Y  K  P  D  E  N  E  V  R  T  L  F  G  L  H  L  S  Q  K  R  N  N  G  V  V     395

gacaagtcattatttagcaatgttgttaccaaaaataaagatttgccagagtctgccctccgagacctcatcgtagccaccattgctgtc    1350
D  K  S  L  F  S  N  V  V  T  K  N  K  D  L  P  E  S  A  L  R  D  L  I  V  A  T  I  A  V     425

aagtacactcagtctaactctgtgtgctacgccaagaacgggcaggttatcggcattggagcaggacagcagtctcgtatacactgcact    1440
K  Y  T  Q  S  N  S  V  C  Y  A  K  N  G  Q  V  I  G  I  G  A  G  Q  Q  S  R  I  H  C  T     455

cgccttgcaggagataaggcaaactattggtggcttagacaccatccacaagtgctttcgatgaagtttaaaacaggagtgaagagagca    1530
R  L  A  G  D  K  A  N  Y  W  W  L  R  H  H  P  Q  V  L  S  M  K  F  K  T  G  V  K  R  A     485

gaaatctccaatgccatcgatcaatatgtgactggaaccattggcgaggatgaagatttgataaagtggaaggcactgtttgaggaagtc    1620
E  I  S  N  A  I  D  Q  Y  V  T  G  T  I  G  E  D  E  D  L  I  K  W  K  A  L  F  E  E  V     515

cctgagttactcactgaggcagagaagaaggaatgggttgagaaactgactgaagtttctatcagctctgatgccttcttcccttccga    1710
P  E  L  L  T  E  A  E  K  K  E  W  V  E  K  L  T  E  V  S  I  S  S  D  A  F  F  P  F  R     545

gataacgtagacagagctaaaaggagtggtgtggcgtacattgcggctccctccggtttctgctgctgacaaagttgtgattgaggcctgc    1800
D  N  V  D  R  A  K  R  S  G  V  A  Y  I  A  A  P  S  G  S  A  A  D  K  V  V  I  E  A  C     575

gacgaactgggaatcatcctcgctcatacgaaccttcggctcttccaccactgattttaccacacactgtttttggcttgcttatgtgt    1890
D  E  L  G  I  I  L  A  H  T  N  L  R  L  F  H  H                                            592

aggtgaacagtcacgcctgaaactttgaggataacttttttaaaaaaataaaacagtatctcttaaaacaatgttttgatctacataaaca    1980

ttgtaaaaattttcaatcacgctttttaactttctttaccacaaaaaaatgataagtgggtgaagtgatggttatgttaattagcgtgc     2068
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Figure 3. Nucleotide and putative amino acid sequences of human *purH* cDNA. Human testis cDNA library was screened by using the radiolabelled PCR product #21 by a plaque hybridization technique. The nucleotide sequence of a cDNA clone with the longest insert was determined. Putative amino acid sequence was deduced by the Genetyx DNA analysis software (SDC, Japan). The nucleotide sequence (underlined) corresponds to that of the fragment recovered by the subtraction procedure described here.

Table 2. Complementation of the *purH* deficiency in the mouse mutants by the human *purH* cDNA clone. Twenty micrograms of plasmid DNA was transfected as the calcium phosphate co-precipitates to the recipient cells. For no DNA control, calcium phosphate co-precipitate without plasmid DNA was exposed to the cells. After one week of cell culture, the number of colonies formed on the two types of plates, with or without adenine, were counted and the transformation frequencies were calculated. pSV2E represents the vector DNA with no insert; pSV2E-HSpurH, with the cDNA insert in the right orientation; and pSV2E-HSpurH rev., with the cDNA insert in the opposite orientation.

	Transformation efficiency		
Plasmid	ade1	ade2	ade3
no DNA	$< 6.0 \times 10^{-7}$	$< 5.0 \times 10^{-7}$	$< 6.5 \times 10^{-7}$
pSV2E	$< 6.2 \times 10^{-7}$	$< 6.0 \times 10^{-7}$	$< 4.1 \times 10^{-7}$
pSV2-HSpurH	5.6×10^{-5}	6.4×10^{-4}	1.5×10^{-4}
pSV2-HSpurH rev.	$< 6.0 \times 10^{-7}$	$< 5.4 \times 10^{-7}$	$< 3.8 \times 10^{-7}$

procedure described here.

Prior to the experiment, we considered the possibility that additional short PCR products are formed by annealing of primers 1 and 2 and reduce the subtraction efficiency. Under the conditions used, however, the formation of such short products was not detected as judged by ethidium bromide staining of agarose gel-solved PCR products (data not shown). Another possibility taken into consideration was that primer 1 carrying a stretch of $(dT)_{10}$ may anneal to various sites of the poly(A) tail, which is usually 100–200 bases long, and generates PCR products of different sizes. As shown in Fig. 2, however, the three independently recovered clones were identical in size. We interpret that as a relatively short extension time was used for PCR, the shortest products became predominant during the cycling reaction.

The minimum length of the 3′-UTR required for RT-PCR amplification and efficient subtraction is not known. If the length between the poly(A) signal and poly(A) tail of the target gene is very short, it may be difficult to apply our procedure. In the case of the human *purH* gene, its length was 128 bases and our subtraction worked successfully. Since known genes with different poly A signal-poly(A) tail lengths are available, it is possible to estimate the minimum length for our subtraction procedure.

The subtractive procedure described here is a powerful tool to clone human housekeeping genes expressed in rodent transformant cells, but it would be difficult to apply to other types of genes, such as those expressed at the particular developmental stages.

On the other hand, housekeeping genes are conserved among different species, both in function and in structure. They can be expressed constitutively to complement the recessive phenotypes in the cultured mutant cells derived from different species. Since advanced procedures for the isolation of recessive mutant cells from rodent cultured cells[1] and subsequent genomic gene transfer techniques using human metaphase chromosomes[1,6] have already been established, combining the subtraction procedure described here with these techniques will facilitate the functional and structural analyses of the human housekeeping genes involved in the various metabolic pathways.

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