Volume 12 Number 6 1984 Nucleic Acids Research

Nucleotide sequence of a full-length cDNA coding for 3-methylcholanthrene-induced rat liver cytochrome P-450MC

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Received 22 December 1983; Revised and Accepted 8 February 1984

ABSTRACT

We constructed a full-length cDNA coding for 3-methylcholanthrene-inducible rat liver cytochrome P-450MC by the method of Okayama and Berg. The isolated clone pAU157 contained the cDNA insert of 2.7 kb in length. Sequence analysis of the cDNA insert revealed that the amino acid sequence of cytochrome P-450MC was composed of 523 amino acid residues, including the initial 22 N-terminal amino acids whose sequence was determined with the purified protein. The primary structure was found to contain two highly conserved regions as pointed out from comparisons of the reported amino acid sequences of cytochrome P-450 species. The predicted molecular weight of the apoprotein was 59,300 daltons. Therefore, we concluded that the amino acid sequence determined here is for cytochrome P-450MC, probably corresponding to cytochrome P-450c.

INTRODUCTION

Cytochrome P-450 in hepatic microsomes is the terminal enzyme of a NADPH-dependent monooxygenase system, which is engaged in oxidative metabolism of a wide variety of structurally unrelated lipophilic drugs and other xenobiotics. The metabolic versatility of the enzyme system results from both the molecular multiplicity of cytochrome P-450 and the broad substrate specificity of individual forms of the enzyme (1,2). Recently, the molecular multiplicity of cytochrome P-450 in rat liver has been well elucidated at the gene or DNA level by using recombinant DNA technology. Fujii-Kuriyama and his collaborators (3,4) reported the complete amino acid sequence of phenobarbital-inducible cytochromes P-450b and P-450e in rat liver by sequence analysis of the cloned cDNAs and the presence of multiple genes for this type of cytochrome P-450 in the rat genome. In addition, Bresnick et al (5) and Fagan et al (6) reported the molecular cloning of cDNA for 3-methylcholanthrene (MC)-inducible cytochrome P-450. However, the restriction maps of these cDNA clones differed each other and sequence analysis of the cDNA inserts has not been reported yet.

Here we report cloning of a full-length cDNA coding for cytochrome P-450MC, which is the major form of MC-inducible cytochrome P-450 in rat liver, sequence analysis of the cloned cDNA, and deduced primary amino acid sequence of cytochrome P-450MC.

MATERIALS AND METHODS

Cytochrome P-450MC and Anti-cytochrome P-450MC IgG

Cytochrome P-450MC, the major form of MC-inducible cytochrome P-450 in rat liver, was isolated from liver microsomes of male Sprague-Dawley rats treated with MC as reported elsewhere (7). The N-terminal sequence of 22 amino acids of the purified enzyme was determined as reported (7). The preparation of rabbit IgG produced with the purified cytochrome P-450MC (anti-cytochrome P-450MC IgG) was also reported (7).

cDNA Cloning

Cytochrome P-450MC mRNA was partially purified from rat liver 14-15 hr after administration of MC as reported elsewhere (8). Poly(A) RNA was prepared from total RNA by two rounds of oligo(dT)-cellulose (PL Biochemicals, Inc.) chromatography and subjected to 10-30 % neutral sucrose density gradient centrifugation. A 16-23S RNA fraction enriched in cytochrome P-450MC mRNA was used for cDNA cloning.

Synthesis of cDNA from partially purified mRNA and cloning of the double-stranded cDNA were performed by the method of Okayama and Berg (9). Bacterial clones of cytochrome P-450MC cDNA were screened by colony hybridization with nick-translated [^{32}P]cDNA prepared from pTZ286, which contained the 800 bp sequence coding for MC-inducible rat liver cytochrome P-450 (8). Recombinant plasmids of positive clones were prepared with an alkali-SDS method (10). All the cloning procedures were conducted in a P-2 biocontainment facility at Takarazuka Research Center in accordance with the guidelines for research involving recombinant DNA molecules issued by the Science and Technics Agency of Japan.

Positive Hybridization Translation Assay

Positive hybridization translation assay was performed as reported elsewhere (8). A recombinant plasmid was bound to a nitrocellulose filter and hybridized with the partially purified mRNA. After extensive washing of the filter, the hybridized mRNA was released and translated in a rabbit reticulocyte lysate system with [^{35}S]methionine as a tracer. The translation product was immunoprecipitated with anti-cytochrome P-450MC IgG coupled to Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals). Immunoprecipitants as

well as total translation products were analyzed by SDS-polyacrylamide gel electrophoresis (11), followed by fluorography (12).

Restriction Enzyme Mapping and DNA Sequencing

Restriction enzyme mapping was carried out using various restriction endonucleases obtained from Takara Shuzo (Japan), Bethesda Research Laboratories, Inc. and New England Biolabs, Inc. The digested DNA fragments were analyzed by electrophoresis on a 0.8-1.0 % agarose gel or 5 % polyacrylamide gel.

DNA sequencing was performed by the procedures of Maxam and Gilbert (13). After the G, G+A, T, and T+C degradation reactions, cleavage products were analyzed on both 8 % and 20 % polyacrylamide gels.

RESULTS

Isolation and Restriction Mapping of Cytochrome P-450MC cDNA Clone

We conducted cloning of a full-length cDNA coding for MC-inducible rat liver cytochrome P-450MC by the method of Okayama and Berg (9). After transformation of *E.coli* DH1 cells with the double-stranded cDNA synthesized from the partially purified mRNA, approximately 2,000 ampicillin-resistant colonies were screened by colony hybridization with the [^{32}P]cDNA probe carrying the 800 bp sequence for MC-inducible cytochrome P-450. Eighteen colonies that hybridized with the probe in different degrees were selected and their plasmid DNAs were isolated, digested with *Pst*I and other restriction endonucleases for determination of the size of inserts. Of these, five clones were verified to contain sequences complementary to cytochrome P-450MC mRNA by positive hybridization translation assay and immunoprecipitation of translation products with anti-cytochrome P-450MC IgG. The *in vitro* translation product of the mRNA hybridized with the plasmid DNA of the positive clone as well as the immunoprecipitant with anti-cytochrome P-450MC IgG proved to be cytochrome P-450MC by SDS-polyacrylamide gel electrophoresis using authentic cytochrome P-450MC.

The clone pAU157 which produced a faint positive signal with colony hybridization was found to have the largest insert of 2.7 kb in length, whereas the other four clones with clear signals contained smaller inserts of 0.8-1.2 kb in length. Cytochrome P-450MC mRNA was estimated to be 22S or approximately 2.7 kb in length on the basis of relative mobility in denatured agarose gels (8). Therefore, the cDNA insert of pAU157 was expected to cover the total length of cytochrome P-450MC.

The insert of pAU157 was mapped with several restriction endonucleases

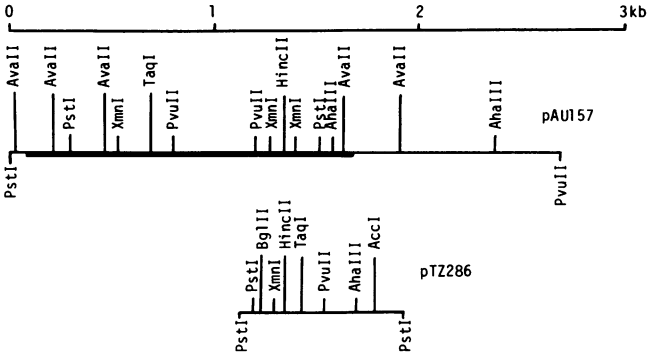

Figure 1. Restriction Endonuclease Maps of pAU157 and pTZ286. The plasmid DNA was digested with several restriction endonucleases and the resulting DNA fragments were electrophoresed on 0.8-1.0 % agarose gel or 5 % polyacrylamide gel. The size of the fragments was determined by comparison to the mobility of size markers. AccI, AvaI, BamHI, BglII, EcoRI, HindIII, NarI, SphI and StuI did not cleave pAU157 within the insert. The bold line in pAU157 represents the coding region of cytochrome P-450MC, as shown in Figure 2.

and compared with the pTZ286 insert as shown in Figure 1. As expected, the restriction map of pAU157 was clearly different from that of pTZ286. In addition, the other four clones contained maps that overlapped with the probe. These results indicated that there exist at least two similar but distinct cDNA clones for MC-inducible cytochrome P-450. Also, the maps of both pAU157 and pTZ286 did not overlap with those of the cDNA clones for MC-inducible cytochrome P-450 reported previously by Bresnick et al (5) and Fagan et al (6).

Sequence Analysis of pAU157 and Primary Amino Acid Sequence of Cytochrome P-450MC

We determined the complete nucleotide sequence of the cDNA insert of pAU157. The sequence of most parts of the region was determined at least twice. The results are shown in Figure 2. Inspection of the nucleotide sequence for location of initiation and termination codons in the three possible reading frames revealed only one open frame of sufficient length to specify a protein of the size of cytochrome P-450MC. The coding sequence occupied 1,569 bp flanked at the 5'-end by 72 bp and the G-C joint, and at the 3'-end by about 1,000 bp plus a poly(A) stretch. In the 3'-noncoding tail, two possible poly(A) addition signals, AATAAA, were present at 502 bp and 18 bp upstream from the poly(A) addition site.

The primary amino acid sequence of rat liver microsomal cytochrome

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                                                                    30
                                  GGGGGGGGGGGGGG CATCCTCCCTGGGGTCCTAGAGAACACTCT

                          60                                       120
TCAGTTCAGTCCTTCCTCACAGCCAAAGCA GCCACCTAGATCATGCCTTCTGTGTATGGA
                                                  ProSerValTyrGly
                                                  1)
                         150                                      180
TTCCCAGCCTTCACATCAGCCACAGAGCTG CTCTTGGCCGTCACCACATTCTGCCTTGGA
PheProAlaPheThrSerAlaThrGluLeu LeuLeuAlaValThrThrPheCysLeuGly

                         210                                      240
TTCTGGGTGGTTAGAGTCACAAGAACCTGG GTTCCCAAAGGTCTGAAGAGTCCACCCGGA
PheTrpValValArgValThrArgThrTrp ValProLysGlyLeuLysSerProProGly

                         270                                      300
CCCTGGGGCTTGCCCTTCATGGGGCACGTG CTGACCCTGGGGAAGAACCCACACCTGTCA
ProTrpGlyLeuProPheMetGlyHisVal LeuThrLeuGlyLysAsnProHisLeuSer

                         330                                      360
CTGACAAAACTGAGTCAGCAGTATGGGGAC GTGCTGCAGATCCGTATTGGCTCCACACCC
LeuThrLysLeuSerGlnGlnTyrGlyAsp ValLeuGlnIleArgIleGlySerThrPro

                         390                                      420
GTGGTGGTGCTGAGCGGCCTGAACACCATC AAGCAGGCCCTGGTGAAACAGGGGGATGAC
ValValValLeuSerGlyLeuAsnThrIle LysGlnAlaLeuValLysGlnGlyAspAsp

                         450                                      480
TTCAAAGGCCGGCCAGACCTCTACAGCTTC ACACTTATCGCTAATGGCCAGAGCATGACT
PheLysGlyArgProAspLeuTyrSerPhe ThrLeuIleAlaAsnGlyGlnSerMetThr

                         510                                      540
TTCAACCCAGACTCTGGACCGCTGTGGGCT GCCCGCCGGCGCCTGGCCCAGAATGCGCTG
PheAsnProAspSerGlyProLeuTrpAla AlaArgArgArgLeuAlaGlnAsnAlaLeu

                         570                                      600
AAGAGTTTCTCCATAGCCTCAGACCCAACA CTGGCATCCTCTTGCTACTTGGAAGAGCAC
LysSerPheSerIleAlaSerAspProThr LeuAlaSerSerCysTyrLeuGluGluHis
                                                  2)
                         630                                      660
GTGAGCAAAGAGGCTGAATACTTAATCAGC AAGTTCCAGAAGCTGATGGCAGAGGTTGGC
ValSerLysGluAlaGluTyrLeuIleSer LysPheGlnLysLeuMetAlaGluValGly

                         690                                      720
CACTTCGACCCTTTCAAGTATTTGGTGGTG TCAGTGGCCAATGTCATCTGTGCCATATGC
HisPheAspProPheLysTyrLeuValVal SerValAlaAsnValIleCysAlaIleCys

                         750                                      780
TTTGGCAGACGTTATGACCACGATGACCAA GAGCTGCTCAGCATAGTCAATCTAAGCAAT
PheGlyArgArgTyrAspHisAspAspGln GluLeuLeuSerIleValAsnLeuSerAsn

                         810                                      840
GAGTTTGGGGAGGTTACTGGTTCTGGATAC CCAGCTGACTTCATTCCTATCCTCCGTTAC
GluPheGlyGluValThrGlySerGlyTyr ProAlaAspPheIleProIleLeuArgTyr

                         870                                      900
CTCCCTAACTCTTCCCTGGATGCCTTCAAG GACTTGAATAAGAAGTTCTACAGTTTCATG
LeuProAsnSerSerLeuAspAlaPheLys AspLeuAsnLysLysPheTyrSerPheMet

                         930                                      960
AAGAAGCTAATCAAAGAGCACTACAGGACA TTTGAGAAGGGCCACATCCGGGACATCACA
LysLysLeuIleLysGluHisTyrArgThr PheGluLysGlyHisIleArgAspIleThr
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                                990                                1020
GACAGCCTCATTGAGCATTGTCAGGACAGG AGGCTGGACGAGAATGCCAATGTCCAGCTC
AspSerLeuIleGluHisCysGlnAspArg ArgLeuAspGluAsnAlaAsnValGlnLeu

                               1050                               1080
TCAGATGATAAGGTCATTACGATTGTTTTT GACCTCTTTGGAGCTGGGTTTGACACAATC
SerAspAspLysValIleThrIleValPhe AspLeuPheGlyAlaGlyPheAspThrIle

                               1110                               1140
ACAACTGCTATCTCTTGGAGCCTCATGTAC CTGGTAACCAACCCTAGGATACAGAGAAAG
ThrThrAlaIleSerTrpSerLeuMetTyr LeuValThrAsnProArgIleGlnArgLys

                               1170                               1200
ATCCAGGAGGAGTTAGACACAGTGATTGGC AGGGATCGGCAGCCCCGGCTTTCTGACAGA
IleGlnGluGluLeuAspThrValIleGly ArgAspArgGlnProArgLeuSerAspArg

                               1230                               1260
CCTCAGCTGCCCTATCTGGAGGCCTTCATC CTGGAGACCTTCCGACATTCATCCTTTGTC
ProGlnLeuProTyrLeuGluAlaPheIle LeuGluThrPheArgHisSerSerPheVal

                               1290                               1320
CCATTCACCATCCCCCACAGCACCATAAGA GATACAAGTCTGAATGGCTTCTATATCCCC
ProPheThrIleProHisSerThrIleArg AspThrSerLeuAsnGlyPheTyrIlePro

                               1350                               1380
AAGGGACACTGTGTCTTTGTGAACCAGTGG CAGGTTAACCATGACCAGGAACTATGGGGT
LysGlyHisCysValPheValAsnGlnTrp GlnValAsnHisAspGlnGluLeuTrpGly

                               1410                               1440
GATCCAAACGAGTTCCGGCCTGAAAGGTTT CTTACCTCCAGTGGCACTCTGGACAAACAC
AspProAsnGluPheArgProGluArgPhe LeuThrSerSerGlyThrLeuAspLysHis

                               1470                               1500
CTGAGTGAGAAGGTCATTCTCTTTGGTTTG GGCAAGCGAAAGTGCATTGGGGAGACCATT
LeuSerGluLysValIleLeuPheGlyLeu GlyLysArgLysCysIleGlyGluThrIle
                          3)
                               1530                               1560
GGCCGACTGGAGGTCTTTCTCTTCCTGGCC ATCCTGCTGCAGCAAATGGAATTTAATGTG
GlyArgLeuGluValPheLeuPheLeuAla IleLeuLeuGlnGlnMetGluPheAsnVal

                               1590                               1620
TCACCAGGCGAGAAGGTGGATATGACTCCT GCCTATGGGCTGACTTTAAAACATGCCCGC
SerProGlyGluLysValAspMetThrPro AlaTyrGlyLeuThrLeuLysHisAlaArg

                               1650                               1680
TGTGAGCACTTCCAAGTGCAGATGCGGTCT TCTGGTCCTCAGCATCTCCAGGCTTAGACT
CysGluHisPheGlnValGlnMetArgSer SerGlyProGlnHisLeuGlnAla***

                               1710                               1740
GTCCTGGATGCTCACCAGACTAGGTGGCTG TTCCTAGGATTCAACTTCAGTCAGAAACAC

                               1770                               1800
AGACCCTGGGGCATTGTGCCTGCCTCCTAC TTTGGACTTGTTTCTCTATATGCTGAACAC

                               1830                               1860
AGACACTGGGCACAGCAGAGACCCACAGGA ACCTCAGATCCTTCTCAAGTTCAGCATCAA

                               1890                               1920
CTAGGAGACCTAAAAGGGTTATGAGATACC TGGGCCTCAGAAAACCCCTGAAGAGCTCTC

                               1950                               1980
TGGTCCTCCAGTGGCTGGCTGGTTTGAAAA ATACTTACAACAGGTCATGCTAGGATCTGG
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                                2010                                2040
CTGGTTACTTTGACAACCGGGAGTAGCCCA GAATGGAGGGAGAAGAGAACTCAAAATACT
                                2070                                2100
GGCACGGAGGTGCTCTTGCCATCTGCTGAG GCTCAACTGTCTTCCAACATGGGTTTATGA
                                2130                                2160
CACTACATGTGGGGGTGTAGCACCTTCATT TACCCTACATAGAAATAAACAAGGTCTCCT
                                2190                                2220
TGTCCTTGCAAAGCCCATGTTCCTGTTTAG GAAGGGCTGAGAGTTGTGTGTAGAAAGACC
                                2250                                2280
TAAGAACATAGGGACAGACTTTCTGGGCAG TAAGACCAGGTTTAGAGTAAAGGAATGCCT
                                2310                                2340
TTTGAGACAGTATTGTGTAGTCCAAGTTGC CTCTGAACTTGCTACCAAGGGTGGCCTTGA
                                2370                                2400
ACTCCTTAATTCTTTTTTCTGCTTTTACCA CCCTACCAAGTGCTAGGGTACAGTCATGAA
                                2430                                2460
CCGCTACACCAGCTCTTGGTCTCTTGTCTT TACTGTATAAAACGTTTCTTTCTTTCTTTT
                                2490                                2520
TTTTTTAAAGAAAATGTTTGTGCATAAGAG TTTTTTATTGTGGCCTGTATTTTGCTTATG
                                2550                                2580
CATTTGTATTAGTCGTACTTCAATAGATTT AGATAATTCGCTTAGTGTAATAGAGAAAAA
                                2610                                2640
TCTAACTCAAGTATCCAGAAATATATAGGA AAAACGTACCTGAGCTAAATAAAAATATTA
                                2670        2700
CCTGGAAAAAAAAAAAAAAAAAAAAAAAA ------AAAAAAAAAAA
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Figure 2. The Nucleotide Sequence of the cDNA Insert of pAU157 and the Deduced Total Amino Acid Sequence of Cytochrome P-450MC. Nucleotide sequence was determined by the method of Maxam and Gilbert (13). The numbers are based on the cloned cDNA and do not indicate the exact location of each nucleotide in the mRNA. The underlined amino acid sequences indicate; 1) the N-terminal amino acid sequence determined with the purified protein, 2) HR1 and 3) HR2, both are highly conserved regions proposed by Gotoh et al (15). Two possible poly(A) addition signals, AATAAA, are also underlined.

P-450MC was deduced from the nucleotide sequence as shown in Figure 2. The single open frame encoded a polypeptide of 523 amino acids, indicating a molecular weight of approximately 59,300 daltons, while the molecular weight of the purified cytochrome P-450MC has been estimated to be 56,000 daltons. In addition, the N-terminal amino acid sequence of 22 amino acid residues of the purified enzyme was in complete agreement with the deduced amino acid sequence. Since cytochrome P-450MC appeared to correspond to cytochrome P-450c in comparison with minimum molecular weights, spectral characteristics, immunochemical properties, and substrate specificities, this sequence was compared with that determined with the purified cytochrome P-450c by Botelho et al (14). In consequence, six amino acid

changes were found in the total 18 amino acid residues of the N-terminal region between both sequences, while the seven C-terminal amino acid sequence deduced from the nucleotide sequence was entirely different from the reported results. Moreover, from a comparison with the reported amino acid sequences of other cytochrome P-450 species, two possible equivalent sequences for the highly conserved regions HR1 and HR2 pointed out by Gotoh et al (15) were found in the amino acid sequence of cytochrome P-450MC: HR1 corresponds to the sequence from the amino acid residues 162 to 176 and HR2 from 453 to 472. Therefore, it was concluded that the amino acid sequence determined here is for cytochrome P-450MC, probably corresponding to cytochrome P-450c.

DISCUSSION

It is known that two molecular species of cytochrome P-450, P-450c and P-450d, are induced by MC-treatment in rat liver, and share common antigenic determinants (16). In the present study, anti-cytochrome P-450MC IgG which had not absorbed with cytochrome P-450d was used for immunochemical identification of translation products of mRNA hybridized with the cloned cDNAs. Therefore, it is necessary to determine which species of MC-inducible cytochrome P-450 mRNA was cloned. The restriction map of the cDNA insert of pAU157 was completely different from that of pTZ286, which was used as a hybridization probe. From a comparison with the results reported recently by Kawajiri et al (17), the map of pTZ286 was found to overlap with that of cytochrome P-450d cDNA clone. On the other hand, sequence analysis revealed that the cDNA insert of pAU157 contained the nucleotide sequence coding for the N-terminal sequence of 22 amino acid residues determined with the purified cytochrome P-450MC (7). The determined N-terminal sequence was completely defferent from that determined with cytochrome P-450d (18), and similar to that of cytochrome P-450c (14), although six amino acid changes were found so far between our determined sequence of cytochrome P-450MC and the reported sequence of cytochrome P-450c. The reason for this discrepancy is not clear. By combining these results, we can conclude that the determined amino acid sequence for cytochrome P-450MC corresponds to cytochrome P-450c and not to cytochrome P-450d.

It appeared that the cloned cDNA contains full- or nearly full-length information of cytochrome P-450MC mRNA, since it consisted of the leader sequence of 72 bp, the coding sequence of 1,569 bp and the 3'-noncoding sequence of approximately 1,000 bp. In addition, the size of cytochrome P-450MC mRNA was estimated from the relative mobility in denatured agarose

gels to be 22S (8), which corresponded to approximately 2.7 kb in length. Therefore, the length of the cDNA insert was thought to be nearly the same as that of cytochrome P-450MC mRNA. Recent results of the primer extention experiment with the AvaII-Sau3AI fragment (56 nucleotides) of pAU157 used as a primer suggested that the cloned cDNA lacks 30 bp in the leader sequence related to the corresponding mRNA (unpublished observations). Also, it is interesting to know that there existed two possible poly(A) addition signals, AATAAA, at 502 bp and 18 bp upstream from the poly(A) addition site. The latter signal appears to actually function in cytochrome P-450MC mRNA, since the signal locates usually about 20 bp upstream from the poly(A) addition site in eukaryotic mRNAs.

Fujii-Kuriyama et al (3) reported that there existed 14 nucleotide substitutions among 922 overlapping nucleotide sequence between two cDNA clones which were complementary to two similar but distinct mRNAs for phenobarbital-inducible rat liver cytochrome P-450, and these base substitutions occurred in a limited portion of the sequence. Therefore, it seems rather difficult to deduce the entire primary structure of certain species of cytochrome P-450 from a combination of small cDNA clones. In the case of cytochrome P-450MC, the complete amino acid sequence was deduced directly from the nucleotide sequence of the cloned cDNA, which contained entirely the full-length information, ranging from the initiation codon to the termination codon. A comparison of the primary structure of cytochrome P-450MC with those of other species revealed that the cytochrome P-450MC sequence contains two highly conserved regions which exhibited high homology with the equivalent sequences of the reported species (15). It would be interesting to know that these conserved regions somehow may be involved in the common feature and biological function of the cytochrome P-450 molecule.

ACKNOWLEDGEMENTS

We express our gratitude to Dr. Fujii-Kuriyama, Japanese Foundation for Cancer Research, for his advice on DNA sequencing. This work was supported by funds obtained under the Research and Development Project of Basic Technologies for Future Industries from the Ministry of International Trade and Industry of Japan.

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