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Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA

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ABSTRACT

Using a set of synthetic oligonucleotides homologous to broadly conserved sequences in-vitro amplification via the polymerase chain reaction followed by direct sequencing results in almost complete nucleotide determination of a gene coding for 16S ribosomal RNA. As a model system the nucleotide sequence of the 16S rRNA gene of M.kansasii was determined and found to be 98.7% homologous to that of M.bovis BCG. This is the first report on a contiguous sequence information of an entire amplified gene spanning 1.5 kb without any subcloning procedures.

INTRODUCTION

The analysis of nucleic acid sequences is central to biology. Until recently the isolation and complete nucleotide determination of genes required cloning or subcloning procedures. A novel development in molecular biology techniques, in-vitro amplification of DNA fragments via the polymerase chain reaction (PCR [1-3]), allows the isolation of a specific gene, thus eliminating the needs for its cloning. However, although several strategies to sequence PCR products directly have been reported (4-9), so far the complete determination of the nucleotide sequence of an entire amplified gene necessitated subcloning procedures (10,11). Due to nucleotide misincorporation, which is inherent for any DNA polymerase, sequencing of cloned amplified DNA may lead to false nucleotide determinations as a result of errors in the PCR (3,10,12,13). These artefacts can be overcome by direct sequencing of the amplified DNA fragment, since all random misincorporations by the enzyme are averaged out (13).

We demonstrate here the isolation and almost complete

nucleotide determination of a gene coding for 16S rRNA using synthetic primers, PCR and direct sequencing of the amplified gene. A peculiarity of genes coding for small subunit ribosomal RNA is that stretches of highly conserved DNA sequences are interspersed among semiconserved and nonconserved sequences (14-16). The highly conserved regions have proven useful as primer target sites for establishing partial sequences derived from direct RNA sequencing (17,18).

In this report we wanted to tackle the problem of defining several oligomer probes that are broadly homologous to conserved eubacterial 16S ribosomal RNA sequences and of demonstrating the usefulness of these synthetic oligomers for amplification and almost complete nucleotide determination of the 16S rRNA gene of Mycobacterium kansasii.

MATERIALS AND METHODS

Mycobacterium kansasii (DSM 43224, Braunschweig, FRG) was grown on Löwenstein-Jensen agar. DNA war extracted according to standard procedures (19).

The following oligonucleotides were used in the PCR and for sequencing (the locations of phylogenetically conserved sequences were identified in comparison of 20 prokaryotic, eubacterial small subunit ribosomal RNA sequences including representatives of the major eubacterial groups [15]; the nucleotide positions of the synthetic DNA oligomers in prokaryotic 16S rRNAs as represented by E. coli are given in brackets):

pA : AGA GTT TGA TCC TGG CTC AG (8- 28);
pB : TAA CAC ATG CAA GTC GAA CG (50- 70);
pC : CTA CGG GAG GCA GCA GTG GG (341-361);
pC•: CCC ACT GCT GCC TCC CGT AG, identical to reverse pC (361-341)
pD : CAG CAG CCG CGG TAA TAC (originally described by Lane et al. [17], 518-536);
pD•: GTA TTA CCG CGG CTG CTG, identical to reverse pD (originally described by Lane et al. [17] 536-518);
pE : AAA CTC AAA GGA ATT GAC GG

(originally described by Lane et al. [17] 908-928);

pE•: CCG TCA ATT CCT TTG AGT TT, identical to reverse pE (originally described by Lane et al. [17] 928-908);

pF : CAT GGC TGT CGT CAG CTC GT (1053-1073);

pF•: ACG AGC TGA CGA CAG CCA TG, identical to reversee pF (1073-1053);

pG•: ACG GGC GGT GTG TAC (originally described by Lane et al. [17] 1407-1392);

pH•: AAG GAG GTG ATC CAG CCG CA (1542-1522)

Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia) and purified by a shadow-casting polyacrylamide gel.

1.0μg of bacterial DNA was subjected to PCR in a total volume of 100μl, with 2.5 units of Taq polymerase (Perkin-Elmer Cetus, Emeryville CA, USA), 50mM KCl, 10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$, 0.01% (w/v) gelatin, 100 picomoles of each of the two primers, 200μM of each dNTP (dATP, dCTP, dGTP, TTP). The 100μl mixture was covered by 100μl of light mineral oil (Sigma #400-5, St. Louis Mo, USA). The thermal profile involved 36 cycles of denaturation at 93°C for 1 min., primer annealing at 37°C for 2 min., and extension at 72°C for 6 min. The DNA was extracted with phenol, precipitated with ethanol, the relevant fragment isolated by agarose gel electrophoresis, electroeluted, purified on Elutip-D columns (Schleicher & Schuell; Dassel, FRG) and ethanolprecipitated. For sequencing, template DNA (0.05-0.2 pmoles; the PCR routinely resulted in 2-4 pmoles of the desired fragment) was annealed to 2.0 pmoles of the respective sequencing primer in 10μl (40mM Tris-HCl pH7.5, 20mM $MgCl_2$, 50mM NaCl; 5 min. 94°C, 2 min. 65°C, 15 min 37°C). The labeling step was carried out for 5 min at 25°C by adding 1.0μl of 0.1M Dithiothreitol, 5.0μCi [α^{-32}P] dCTP, 2.0μl labeling nucleotide mix (dGTP, dATP, TTP at 200nM) and 3 units sequenase (USB), total volume was 15.0 μl. 3.5μl of this reaction were added to 2.5μl of the appropriate termination mix (80μM dNTP's, 8μM ddNTP, USB kit) and incubated for 5 min. at 37°C. 4μl of formamide/dye stop mix were added and heated for 5 min. at 94°C before loading 2.5μl on a 6% sequencing gel (19). After electrophoresis gels were fixed in 10% acetic

acid, 12% methanol, dried and exposed to X-ray films for 12 hrs.

RESULTS

Published 16S rRNA sequences (15) from phylogenetically diverse eubacteria were searched for regions of sequence conservation to devise oligonucleotides suitable for amplification as well as sequence determination of almost the entire gene. Although oligonucleotides complementary to such structures have been described (17), they were found not to meet the demands needed. First of all they would only allow amplification of parts of the 16S rRNA gene since probes in the 5' and very 3' distal part of the gene were lacking. Secondly, the known oligonucleotides would not allow to obtain contiguous sequence information. Therefore, after additional sequence comparisons we designed five synthetic DNA oligonucleotides complementary to conserved regions in eubacterial 16S rRNA gene sequences. The gene sequences and their conservations in a phylogenetically broad range of eubacteria are shown in Table 1. A scheme of the primers used for our studies is depicted in Fig.1.

The two oligonucleotides designed for use as flanking primers in the enzymatic amplification reaction span a DNA fragment which consists of almost the entire 16S rRNA gene (corresponding to E.coli 16S rRNA positions 8 to 1542). As can be seen in Fig.2 the oligonucleotides directed the synthesis of a 1.5 kb DNA fragment. In each case tested the appropriate DNA fragment was successfully amplified for quite a number of phylogenetically distinct bacteria, such as Proteus vulgaris, Legionella pneumophila, Pseudomonas picketii, Mycobacterium avium, Hemophilus species, Escherichia coli and Nocardia asteroides. It should be noted that the appropriate DNA fragments were amplified for species whose sequences were not used in the design of the probes (L. pneumophila, Ps. picketii, M. avium, H. species) thus demonstrating the conserved nature and broad applicability of the primers used.

As a model system we have chosen to apply this strategy to study the 16S rRNA gene of M.kansasii. Using two primers for

Table 1: rRNA sequences complementary to the oligonucleotide probes. Oligonucleotide sequences are written 5' to 3'. For uniformity, uridine residues are changed to thymidines. Alternate bases are shown below each probe sequence at their respective sites. N refers to undetermined nucleotides.

		primer A	primer B
		AGA GTT TGA TCC TGG CTC AG	TAA CAC ATG CAA GTC GAA CG
Agrobacterium tumefaciens	(A. t.)	... ...	... ...
Rochalimea quintana	(R. q.)	... ...	... ...G ..
Pseudomonas testosteroni	(Ps.t.)	... ...	... ...
Escherichia coli	(E. c.)	... ...A	... ...
Proteus vulgaris	(P. v.)	... ...A	... ...G ..
Ruminobacter amylophilus	(R. a.)	... ...A	... T.. ... ...
Wolinella succinogenes	(W. s.)	... ...	... T.. ... ...
Desulfovibrio desulfuricans	(D. d.)	... ...	... ...
Myxococcus xanthus	(M. x.)	... ...	... ...G ..
Bacillus subtilis	(B. s.)	... ...	... T.. ... ...G ..
Heliobacterium chlorum	(H.ch.)	NNN NNN N..	... ...
Mycoplasma capricolum	(M. c.)	... ...	... T.. ... ...
Mycoplasma sp.	(M.sp.)	... ...	... T.. ... ... C.. ... ..
Mycoplasma hyopneumonial	(M. h.)	... ...	... T.. ... ...
Streptomyces coelicolor	(S. c.)	... ...	... ...
Bacteroides fragilis	(B. f.)	... ...	... ...G G.
Flavobacterium heparinum	(F. h.)	... ...	... T.. ... ...
Thermomicrobium roseum	(T. r.)	... ...	... TG. ... ...
Thermotuga maritima	(T. m.)	..G	... ...G ..
Anacystis rideslans	(A. r.)	... ...	... ...

	primer C	primer F	primer H'
	CTA CGG GAG GCA GCA GCA GTG GG	C ATG GCT GTC GTC AGC TCG T	AAG GAG GTG ATC CAG CCG CA
(A. t.)	... ...	. ...	... ...
(R. q.)	... ...	. ...	... ...A ..
(Ps.t.)	... ...	. ...	... ...
(E. c.)	... ...	. ...	... ...A ... ..
(P. v.)	... ...	. ...	... ...A ... ..
(R. a.)	... ...A ..	. ...	... ...A ..A ..
(W. s.)	... ...A ..	. .C.	... ... NNN NNN NN
(D. d.)	... ...	. ...	... ...
(M. x.)	... ...	. ...	... ...
(B. s.)	... ...A ..	. ... .T.	... ...
(H.ch.)	... ...	. ... .T.	... ...
(M. c.)	... ...A ..	. ... .T.	... ...T ... ..
(M.sp.)	... ...A ..	. ... .T.	... ...T ... ..
(M. h.)	... ...A A.	. ... .T.	... ... T.. ..T .C. ..
(S. c.)	... ...	. ...	... ...
(B. f.)	... ... A.	. ... .T.	... ... T.. ... .
(F. h.)	... ...A A.	. ...	... ... T.. NNN NNN NN
(T. r.)	... ... .CA ..	. ...	... ...C .NN NN
(T. m.)	... ...	. .C. ..C	... ...
(A. r.)	... ...	. ...	... ...A ..

PCR and a set of 12 primers for direct sequencing of the amplified gene the almost entire nucleotide sequence of the 16S rRNA gene of M.kansasii was determined (see Fig. 3), 40% of which sequence information of both strands was obtained. From a given primer site approximately 200 to 250 bases could be resolved where the first readable bases were 5 to 20 nucleotides from the 3' end of the sequencing primer. The 16S rRNA gene of M.kansasii was found to be 98.7% homologous to that of M.bovis BCG (20).

The procedure outlined in this report is much more accurate than direct rRNA sequencing by reverse transcriptase

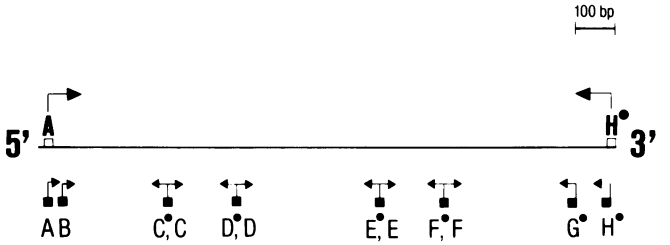

Fig. 1: Sequencing strategy and physical map of the primers used with respect to their target sites in the 16S RNA gene. Primers directing sequence extension in 3' to 5' direction are characterized by a dot •. Primers used for PCR are characterized by an open box, and the direction of the polymerase mediated extension is indicated by an arrow. Primers used for sequence determination are depicted by a closed box, and the direction of sequence extension is indicated by an arrow.

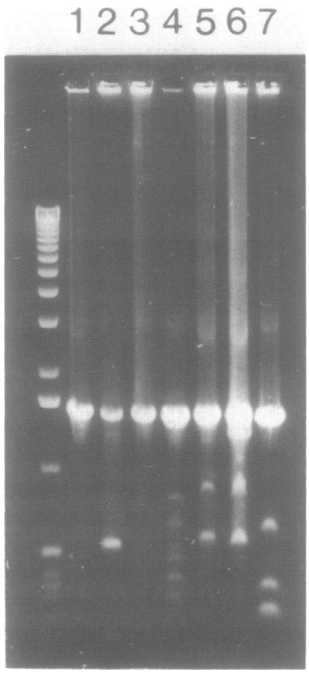

Fig. 2: PCR-mediated synthesis of the 16S rRNA gene spanning 1500 bp of Proteus vulgaris, clinical isolate (1), Legionella pneumophila ATCC 33153 (2), Pseudomonas picketii, clinical isolate (3), Mycobacterium avium, clinical isolate (4), Hemophilus species, clinical isolate (5), Escherichia coli ATCC 25922 (6) and Nocardia asteroides ATCC 3306 (7). Primers used were pA und pH•. Molecular weight marker is the 1 kb ladder (BRL, Gaithersburg, USA).

GCGGCGTGCT TAACACATGC AAGTCGAACG GAAAGGTCTC TTCGGAGACA CTCGAGTGGC
GAACGGGTGA GTAACACGTG GGCAATCTGC CCTGCACACC GGGATAAGCC TGGGAAACTG
GGTCTAATAC CGGATAGGAC CACTTGGCGC ATGCCTTGTG GTGGAAAGCT TTTGCGGTGT
GGGATGGGCC CGCGGCCTAT CAGCTTGTTG GTGGGGTGAC GGCCTACCAA GGCGACGACG
GGTAGCCGGC CTGAGAGGGT GTCCGGCCAC ACTGGGACTG AGATACGGCC CAGACTCCTA
CGGGAGGCAG CAGTGGGGAA TATTGCACAA TGGGCGCAAG CCTGATGCAG CGACGCCGCG
TGGGGGATGA CGGCCTTCGG GTTGTAAACC TCTTTCACCA TCGACGAAGG TCCGGGTTCT
CTCGGATTGA CGGTAGGTGG AGAAGAAGCA CCGGCCAACT ACGTGCCAGC AGCCGCGNTA
ATACGTAGGG TGCGAGCGTT GTCCGGAATT ACTGGGCGTA AAGAGCTCGT AGGTGGTTTG
TCGCGTTGTT CGTGAAATCT CACGGCTTAA CTGTGAGCGT GCGNGCGATA CGGGCAGACT
AGAGTACTGC AGGGGAGACT GGAATTCCTG GTGTAGCGGT GGAATGCGCA GATATCAGGA
GGAACACCGG TGGCGAAGGC GGGTCTCTGG GCAGTAACTG ACGCTGAGGA GCGAAAGCGT
GGGGAGCGAA CAGGATTAGA TACCCTGGTA GTCCACGCNG TAAACGGTGG GTACTAGGTG
TGGGTTTCCT TCCTTGGGAT CCGTGCCGTA GCTAACGCAT TAAGTACCCC GCCTGGGGAG
TACGGCNGCA AGGCTAAAAC TCAAAGGAAT TGACGGGGGN CCGCACAAGC GGCGGAGCAT
GTGGATTAAT TCGATGCAAC GCGAAGAACC TTACCTGGGT TTGACATGCA CAGGACGCGT
CTAGAGATAG GCGTTCCCTT GTGGCCTGTG TGCAGGTGGT GCATGGCTGT CGTCAGCTCG
TGTCGTGAGA TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTTGTCTCA TGTTGCCAGC
GGGTAATGCC GGGGACTCGT GAGAGACTGC CGGGGTCAAC TCGGAGGAAG GTGGGGATGA
CGTCAAGTCA TCATGCCCCT TATGTCCAGG GCTTCACACA TGCTACAATG GCCGGTACAA
AGGGCTGCGA TGCCGCGAGG TTAAGCGAAT CCTTTTAAAG CCGGTCTCAG TTCGGATCGG
GGTCTGCAAC TCGACCCCGT GAAGTCGGAG TCGCTAGTAA TCGCAGATCA GCAACGCTGC
GGTGAATACG TTCCCGGGCC TTGTACACAC CGCCCGTCAC GTCATGAAAG TCGGTAACAC
CCGAAGCCAG TGGCCTAACC CTCGGGAGGG AGCTGTCGAA GGTGGGATCG GCGATTGGGA
CGAAGTCGTA ACAAGGTAGC CGT

Fig. 3: Nucleotide sequence of the 16S rRNA gene of M.kansasii. The noncoding (RNA-like) strand is shown. The sequence comprises 1463 nucleotides (about 96% of the E.coli 16S rRNA) lacking the very proximal 5' and terminal 3' regions, corresponding to E.coli 16S rRNA positions 37 and 1506 respectively. N refers to undetermined nucleotides.

(17) and while direct rRNA sequencing only yields partial sequence information (17,18), the herein described method allows nearly complete and contiguous sequence determination, so far possible only by using traditional molecular biology techniques, e.g. cloning, subcloning and sequencing rRNA genes.

DISCUSSION

In-vitro amplification technology has become a powerful tool for fast and precise production of DNA fragments or even entire genes, and the importance of PCR for the desired applications is based on the circumvention of cloning procedures. Although PCR has been known to allow the isolation of entire genes, to date the complete nucleotide sequence determination of an entire amplified gene required subcloning procedures (10,11). Sequencing of cloned amplified DNA, however, may result in false nucleotide determination due to misincorporations by the polymerase during the amplification reaction (3,10,12,13). These artefacts can only be resolved by

sequencing a statistical number of clones. Another possibility to overcome the problem of misincorporation during PCR is to directly sequence the amplified DNA fragment. The present paper is, to our knowledge, the first report demonstrating a contiguous sequence determination of an almost entire amplified gene spanning 1.5 kb without any subcloning procedures.

As a model system we have isolated and determined the complete nucleotide structure of a gene coding for 16S rRNA of M.kansasii using synthetic primers. Genes coding for small subunit ribosomal RNA are characterized by stretches of highly conserved DNA sequences interspersed among semiconserved and nonconserved sequences (14-16), and similar features are offered by other gene families, e.g. genes coding for immunoglobulines. The conserved eubacterial 16S ribosomal RNA sequences allowed the construction of several oligonucleotides that are broadly homologous to such conserved DNA structures (17, and Table 1). Particular useful for our approach were primers pA and pH$^{\bullet}$ which flank the extreme 5' and 3' part of the 16S ribosomal RNA gene, thus allowing enzymatic amplification of the entire gene. To our surprise the known strong secondary structure of rRNA (16) did not prevent the amplification of its entire gene. The contiguous sequence determination gathered by direct sequencing of the amplified gene was made possible by the additional construction of oligonucleotides used for the sequencing reactions, which are complementary to broadly conserved sequence structures (primers pB, pC and pF), since sequencing the amplified DNA fragment with published "universal" primers (pD, pE, pG$^{\bullet}$) only resulted in partial sequence information. The utility of the primers described in this report, used for PCR or for sequencing, has been tested in enzymatic amplification reactions with DNA templates from over 30 organisms representing 8 different eubacterial genera, and no DNA tested has failed to serve as a template.

The rapidity of the described procedure (from cell pellet to nucleotide sequence determination in approximately 3 to 4 days) compares favourably to the 3 to 6 months required to

clone and sequence a rRNA gene by traditional molecular biology techniques. The outlined procedure allows approximately 40% of the sequence to be determined from both strands and the nucleotide determination is much more accurate than direct rRNA sequencing where the frequency of misidentifying or omitting a residue is approximately 5% (21). The poor reliability of rRNA sequencing using reverse transcriptase requires alignement procedures with published sequences as well as secondary structure models and comparisons with oligonucleotide catalogues (22) to search for sequencing artefacts. In contrast to direct rRNA sequencing which only yields partial sequence information the combination of PCR with the herein described broadly applicable oligonucleotides allows the almost complete and contiguous sequence determination of genes coding for eubacterial 16S rRNA. Another drawback in direct ribosomal RNA sequencing using reverse transcriptase is the requirement for considerable amounts of rRNA and hence the necessity of large numbers of the bacteria of interest (17). Due to the difficulty of obtaining intact rRNA from bacteria (bacteria are notoriously RNase rich) the quality of the rRNA may significantly vary thus resulting in considerable fluctuations of the amount of sequence information gathered. In contrast, DNA suitable for PCR is much easier to obtain and even minimal amounts of DNA are sufficient for the amplification reactions (1-3).

The principle of using rRNA sequences to characterize micro-organisms has gained wide acceptance (23,24). The approach described here rapidly provides complete, unambiguous and contiguous sequence determination of 16S rRNA. The analysis of nucleic acid sequences coding for 16S rRNA is particular useful for phylogenetic analysis (17,18,21,25) and for characterization of an organism of uncertain affiliation. Furthermore, the variable domains of the 16S rRNA molecule show considerable sequence diversity between closely related species (16) and can therefore be used as target sites for construction of taxon-specific probes for rapid identification of microorganisms (26-29). The strategy used in our approach,

i.e. the search for conserved nucleic acids structures in gene families as primer target sites for PCR and direct sequence determination provides a rapid and reliable sequence determination of amplified genes.

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