

RESEARCH NEWS

Nucleic acids: the leading edge

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Recombinant-DNA techniques have become indispensable tools in molecular biology. One of the recent techniques, viz. polymerase chain reaction (PCR), is revolutionizing the entire field. It is so powerful that cloning and expression of any gene of interest takes only days and not months or years. PCR technology has also been used successfully in diagnosis of pathogens such as bacteria, viruses and parasites. Another new development is antisense technology. Antisense RNA and/or DNA oligonucleotides are used to control specifically the expression of a particular gene. Antisense technology relies on the synthesis of modified oligonucleotides that can penetrate the cell and also resist the action of endogenous nucleases. Recent advances in these front-line areas were discussed in a meeting at San Diego, USA (20-22 November 1991), organized by the American Association for Clinical Chemistry. It was one of the rare meetings in which more than eighty per cent of the participants (invited speakers and poster presenters) were from industries involved in applying various aspects of nucleic-acid chemistry in research.

PCR involves thermal cycling in which denaturation of the template DNA is carried out at a very high temperature (93°C), annealing of the oligonucleotide primers to the single-stranded templates is carried out at a moderate temperature (a few degrees below the T_m of the primers), and extension of the primers is carried out at 72°C (optimum temperature for *Taq* DNA polymerase). Repeating this process of denaturation, annealing and extension many times results in the amplification of the template a million

fold. PCR finds immense use in the cloning of genes and in diagnostics (R. Saiki, Cetus Corporation, Emeryville, USA).

An alternative technology, viz. ligase chain reaction (LCR), which finds use only in diagnostics, was also discussed (K. Backman, Omnigene, Cambridge, USA). In LCR, the reaction mixture contains four primers representing adjacent sequences on two complementary strands and a thermostable DNA ligase. The processes of denaturation, annealing and joining are repeated in many cycles. The result of each ligation event is an approximate doubling of target-equivalent molecules. Repetition of this process results in an exponential accumulation of target-equivalent molecules.

Efforts are being made to develop isothermal amplification methods. One such method is nucleic acid sequence-based amplification (NASBA). Three enzymes, viz. reverse transcriptase, RNase H and T7 RNA polymerase, and two specific primers, P1 (which also contains a T7 promoter sequence) and P2, work together to amplify a specific nucleic-acid sequence in excess of 10^8 fold. This method has been successfully used in diagnosis of the human immunodeficiency virus HIV-1 (L. Malek, Cangene Corporation, Mississauga, Canada).

An attractive strategy for detecting infectious organisms is devised by joining a probe sequence with the sequence of a replicatable RNA which can be amplified exponentially after hybridization. The usefulness of this approach is demonstrated by embedding a probe sequence (say HIV-1) within the sequence of a replicatable RNA of the *Q β* virus

These recombinant RNAs hybridize to target nucleic acids as do ordinary probes and these probe-target hybrids are then separated from the nonhybridized probes by capture hybridization. These captured recombinant RNAs can be amplified exponentially by incubation with *Q β* replicase (an RNA-dependent RNA polymerase) and then detected by usual procedures (F. R. Kramer, Public Health Research Institute, New York, USA).

One of the persistent problems associated with PCR is carry-over or cross-contamination. This can result in a number of false positives in diagnosis. Two existing, but inefficient methods for overcoming this problem are the enzymatic method and the chemical method. In the enzymatic method, dUTP is substituted for dTTP in the extension reaction. Thus, after PCR, the reaction product is treated with uracil DNA glycosylase, which cleaves at the dUTP residues, and thus carry-over of the PCR product is prevented. In the chemical method, the PCR product is treated with IP-10, an isopsoralen derivative. Upon exposure to UV light, IP-10 reacts with pyrimidine bases and forms cyclobutane adducts. This modified PCR product is refractory to amplification by *Taq* DNA polymerase. An efficient, cost-effective alternative method is the Triple-C primer method (Triple-C stands for carry-over contamination control). These novel primers contain a 3' ribose residue, the extension of which generates a cleavable RNA linkage. The cleavage of primers from the amplicon either with base or with RNase prevents further amplification (S. D. Harmon, Integrated DNA Technologies, Coralville, USA).

A major obstacle in the use of these amplification technologies in diagnostics is the difficulty associated with analysis of the amplified products using agarose gel electrophoresis. A novel method has been developed by coupling PCR and electrochemiluminescence (ECL). In this method, one of the PCR primers carries biotin at the 5' end. A third oligomer labelled with ORIGEN (an *N*-hydroxy-succinimide ester of Ru II label), which can bind to the strand containing the biotin label, is used in the PCR reaction. After the reaction is completed, the strand carrying the biotin label, which is already hybridized to the oligomer carrying the ORIGEN label, is captured using streptavidin-coated magnetic beads. The light generated by these hybrids can then be easily detected (S. Gudibande, IGEN, Rockville, USA). Another attractive method is enzymatic detection of PCR (ED-PCR), wherein one of the PCR primers carries a biotin label and the other carries a hapten. This method is based on capture of amplified products via biotin-streptavidin affinity and detection of an incorporated hapten in the amplified products with enzyme-tagged antibody. This method has been successfully used in detection of methicillin-resistant *Staphylococcus aureus* (S. Nakagami, Wakunaga Pharmaceutical Company, Hiroshima, Japan).

Other DNA diagnostic methods, not based on PCR amplification, have been developed. A novel method for rapid analysis of sequence variations in genetic defects combines dideoxy sequencing with d'oxigenin (DIG)-labelled primers and detection of the sequencing-reaction products, after electrophoresis and transfer onto a positively charged nylon membrane, using anti-DIG antibody coupled to alkaline phosphatase (C. Kessler, Boehringer-Mannheim, Penzberg, Germany). Another method is Ampliprobe-one step. This method is based on hybridization of target-specific primary probe to the target sequence, followed by hybridization of a target-independent, enzyme-linked secondary probe to common regions of the primary probe (L. A. Ojert, ImClone Systems, New York, USA).

PCR and other, related amplification strategies are always not suitable for use in epidemiological surveys and mass diagnosis for reasons of cost-effective-

ness and feasibility. A novel nonradioactive DNA diagnostic method for detection of *Plasmodium falciparum* infection in blood samples has been developed using a single oligonucleotide probe, whose sequence is repeated many times in the parasite's genome. In this method, a drop of blood from a finger prick is lysed and parasite DNA is hybridized to biotinylated oligonucleotide probe in solution. The hybrid DNA is captured by the oligonucleotide immobilized on microtitre plates. The biotinylated oligonucleotide retained on the plate is assayed by streptavidin-alkaline phosphatase conjugate. This method can be of general application for detection of any pathogen in blood or other body fluids (K. Ayyanathan and S. Datta, Astra Research Centre India, Bangalore, India). A novel strategy for detection of human hepatitis B and C virus particles directly in human serum or plasma makes use of branched oligonucleotides that are designed to contain a unique primary segment and a set of identical secondary fragments covalently attached to the primary sequence through branch points. These secondary fragments serve to amplify the signal in hybridization assays (M. S. Urdea, Chiron Corporation, Emeryville, USA).

Antisense technology relies on development of new chemistries for synthesis of modified nucleotides. Efforts are being made towards developing analogues that bind effectively to specific DNA or RNA targets and yet are more resistant to nucleases or more efficiently taken up by cells than their natural counterparts. Synthesis and hybridization properties of a number of oligonucleotides bearing modifications of the phosphorus backbone have been reported. These analogues include phosphorothioates, phosphoramidates, phosphotriesters and oligomers with cationic or highly lipophilic substituents such as cholesterol. It was observed that hybridization kinetics was altered by cationic substituents and that inhibition of HIV in cell culture was augmented by a cholesteryl substituent (R. L. Letsinger, North-Western University, Evanston, USA).

Nonionic oligonucleotide analogues with methyl phosphonate backbone have been synthesized and studied extensively for duplex stability, pharma-

cokinetics and other parameters. This family of modified oligonucleotides is known by the acronym Matagen, i.e. masking tape for gene expression. One specific application of Matagen in biological systems was illustrated by showing inhibition of herpes simplex virus (HSV-1) (P.O.P. Ts'0, Johns Hopkins University, Baltimore, USA). Considerable progress has been made in synthesis of modified oligonucleotides, e.g. methyloxodeoxycytidine analogues (MODA), which have enhanced biological efficiency. These analogues have been shown to exhibit efficient strand switching (3'-3' switchback) recognition and inhibition of transcription at various pH values. In combination with alkylators such as formacetal, these modified oligonucleotides yield cross-linked target DNA (M. D. Matteucci, Gilead Sciences, Foster City, USA).

Modified oligonucleotides find use in selective inhibition of gene(s) in biological systems. One specific example is the antisense inhibition of *ras* p21 expression. A single point mutation in the 12th or 61st amino-acid coding region transforms the normal *c-ras* protooncogene. Efforts were made to revert the transformed phenotype of tumour cells using anti-*ras* methyl phosphonate oligonucleotides (ONMPs), which were designed to interact with *ras* mRNA specifically at the 12th amino acid or at the 61st amino acid coding region. The inhibitory effects of these oligonucleotides were monitored in a rabbit reticulocyte lysate *in vitro* translation experiment with *ras* transcripts as templates. At 150 μ M concentration of these two oligonucleotides there was greater-than-95% inhibition of synthesis of p21 protein (E. H. Chang, Uniformed Services University of the Health Sciences, Bethesda, USA).

Though dramatic improvements have been made in these nucleic-acid techniques, viz. amplification and antisense technology, in the recent past, they still remain in the realm of laboratory practice. Considerable efforts should be made to simplify these techniques so that they can be efficiently used to diagnose diseases and control them.

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