Diheteroduplex formation using gold labeled single-stranded PCR fragments and its application in electron microscopy

C.-Thomas Bock, Susanne Schwinn, Hanswalter Zentgraf

German Cancer Research Center, Applied Tumor Virology, INF 242, D-69120 Heidelberg, Germany

Received: 5 January 1995; in revised form: 13 February 1995 / Accepted: 23 February 1995

Abstract. Heteroduplex analysis is commonly used to map homologous sequences in DNA:DNA or DNA:RNA hybrids in spread preparations by electron microscopy. However, the standard procedures are not suitable to detect the orientation of a fragment with a defined sequence in a hybrid molecule. Here, we describe an alternative protocol for the visualization of DNA:DNA "diheteroduplex" structures based on digoxigenin/anti-digoxigenin gold labeling that allows determination of the position and orientation of a fragment. Single-stranded polymerase chain reaction (PCR) generated fragments labeled at their 3' ends are hybridized to double-stranded plasmid DNA. Electron microscopy of spread preparations visualizes the gold label and, in combination with morphometric measurements, it is possible to determine the position and orientation of the fragment with the diheteroduplex molecule.

Introduction

Davis and Davidson (1968) and Westmoreland et al. (1969) were able to extend the classical spreading techniques for double-stranded DNA molecules to single-stranded molecules of DNA and RNA. This opened the field for heteroduplex formation (Davis et al. 1971) and the R-loop formation technique (Thomas et al. 1976; White and Hogness 1977). The R-loop method allows mapping of DNA sequences complementary to specific RNA molecules. The heteroduplex method is based on DNA:DNA hybridization and allows detection and mapping of regions of homology between two DNA molecules. A modification of the method is the formation of a triple hybrid, also called heterotriplex, double heteroduplex, or diheteroduplex, a structure formed by hybridizing three different molecules. This triple hybrid may contain either three DNA, or DNA and RNA molecules. Only hybrids consisting of three different DNA molecules should be termed "diheteroduplex" whereas the more general term "triple hybrid" should be used for mixed RNA:DNA hybrids (Brack 1981).

The limitations imposed by the protein monolayer technique on the resolution of small hybrid regions along a DNA molecule have prompted several groups to develop methods for indirect visualization of these structures. These "tagging" methods are based on the strategy of attaching an electron dense label directly or indirectly to the target site. In the first attempt the electron dense protein ferritin was coupled directly to the 3' end of RNA molecules (Wu and Davidson 1973). An alternative method for attaching ferritin to RNA in triple hybrids has been described by Angerer et al. (1976) and Broker et al. (1978). It is based on the high affinity interaction of the egg-white protein avidin with biotin and results in a biotin-RNA:DNA hybrid with ferritin-avidin. A different method, which is based on the enzymatic incorporation of biotin-labeled nucleotides into the ends of restriction fragments and the binding of such biotin-labeled DNAs to avidin-agarose columns has been described by Delius et al. (1985). After strand separation the terminal biotin label is visualized by complex formation with a streptavidin-ferritin conjugate.

Here, we describe end-labeling of DNA molecules with a gold tag. The protocol is based on 3' end-labeling of single-stranded polymerase chain reaction (PCR) products with digoxigenin-conjugated dUTP (dig-dUTP) followed by binding of anti-digoxigenin gold-conjugated antibodies. This protocol seems to be simpler than the previously described tagging methods. Furthermore, it is not restricted to fragments obtained after enzymatic digestion since PCR allows the generation of any DNA fragment. To establish our method, we have generated single-stranded PCR products specific for the genome of hepatitis B virus (HBV; for HBV review see Tiollais et al. 1985; Ganem and Varmus 1987). Annealing the gold-tagged PCR product provided position and orientation markers for the electron microscopic analysis of resulting diheteroduplexes.
Materials and methods

Plasmids. Plasmid 4al (p4al; Loncarevic et al. 1990) contains the XhoI monomer of HBV wild-type DNA, plasmid d4al (pd4al; Schranz et al. 1990) a dimerized HBV genome in head-to-tail orientation, both inserted into the Bluescript vector KS- (Stratagene). Plasmid DNAs were prepared by alkaline lysis (Maniatis et al. 1982), purified by a single round of cesium chloride/ethidium bromide gradient centrifugation and extracted with n-butanol followed by ethanol precipitation. DNA concentrations were determined by spectrophotometric measurement at 260 nm (Ultraspex, Pharmacia LKB).

PCR amplification of single-stranded DNA molecules. Single-stranded PCR fragments 1 and 2 (Fig. 1) were amplified from p4al DNA according to the protocol of Gyllensten and Erlich (1988), with the following modifications. To generate PCR fragment 1 we synthesized oligonucleotide primers that were specific for the HBV DNA. The sequences of this pair of primers (06/19, CTTGAACAGTAGGACATGA, antisense), flanking the HBV polymerase-/X region. HBV specific fragment 2 primers (MD27, CGAGCAAGAACAGAGACATGA, antisense; MD31, antisense), have been published previously (Larzul et al. 1990) and flanked the core region of the HBV genome (Fig. 1). For PCR amplification, the template DNA (p4al; 300 ng) was added to a 100 µl reaction mixture that contained the four dNTPs (each at 200 µM), 2 mM MgCl₂, 0.08% Nonidet-P 40, 2 U Taq DNA polymerase (MBI Fermentas, Lithuania) and 100 pmol of primer 1 (sense primer) and 2 pmol of primer 2 (antisense primer), respectively. PCR was performed in a DNA Thermocycler (Perkin-Elmer-Cetus). The first round of PCR included one cycle for 1 min at 92 °C, 1 min at 58 °C and 2 min at 72 °C and a final incubation for 1 min at 92 °C. PCR products were purified by extraction with a 1:1 mixture of buffer-saturated phenol (10 mM Tris-HCl, pH 7.2) and chloroform (v/v) and were then precipitated with 2.5 vol ethanol containing 300 mM sodium acetate (pH 5.4). To generate the single-stranded PCR product, a 10 µl aliquot of the first PCR product was used as template in a second PCR reaction using only primer 1 (sense) and identical cycling conditions as described for the first round. The final PCR product was phenol/chloroform extracted and ethanol precipitated. The purity of the amplified single-stranded DNA was determined by agarose gel electrophoresis.

Hybridization procedure. Hybrid molecules between dig-dUTP/anti-dig gold-labeled single-stranded DNA and Scal-linearized double-stranded plasmid DNA (p4al; pd4al) were generated using a modification of the protocol described by Westmoreland et al. (1969). Briefly, 5 µl plasmid DNA (100 ng/µl) was added to 15 µl denaturation buffer (0.1 N NaOH, 20 mM trisodium EDTA). After denaturation for 10 min at room temperature, 5 µl 2 M Tris-HCl, pH 7.2), 10 µl 100% formamide and 2 µl of the dig-dUTP/anti-dig gold-labeled PCR fragment were added. The mixture was allowed to renature for 1.5 h at 24 °C and finally mixed with an equal volume of 100% formamide.

Electron microscopy of spread preparations. Spread preparations of single-stranded anti-dig gold-labeled DNA molecules were made according to the "on drop" procedure using cytochrome c (Bock and Zentgraf 1993). Spreading of hybrid molecules was performed following a protein-free spreading technique described recently (Bock et al. 1994). Specimens were finally shadowed with platinum/palladium (80/20) at an angle of 8° for contrast enhancement.

With this report, we introduce a new technique for the visualization of diheteroduplex molecules by electron microscopy. The first step in this protocol is the generation of a sequence-specific single-stranded DNA fragment, trisodium-EDTA, pH 8.0 was added to a final concentration of 20 mM and the reaction mixture chilled on ice. The tailed fragments were precipitated with ethanol without prior phenol extraction.

Gold labeling of dig-dUTP labeled DNA molecules. The dig-dUTP labeled DNA was resuspended in 20 µl binding buffer (200 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100, pH 8.0) and incubated with polyclonal sheep anti-dig/gold-conjugated antibodies [diluted 1:50 in phosphate buffered saline (PBS) pH 7.2, gold size 5 nm; Serva, Heidelberg] for 1.5 h at 37 °C. To remove excess unbound antibodies, the mixture was passed through a Sephadex 2CL/B column prepared in a Pasteur pipet (4x0.5 cm; 300 µl bed volume) and equilibrated with 4 mM magnesium acetate containing 0.01% glutardialdehyde, pH 8.0. Ten fractions (100 µl) were collected and the DNA content of each fraction was determined by the agarose plate method (Maniatis et al. 1982). Glutardialdehyde was added to the DNA containing fractions at a final concentration of 0.2% (v/v) and fractions were incubated for 15 min at 4 ° C. The efficiency of labeling was routinely controlled by electron microscopy of spread preparations.

Results and discussion

Fig. 1. Schematic diagram depicting diheteroduplex formation between single-stranded polymerase chain reaction (PCR) fragments 1 and 2 (dotted bars 1 and 2) and the double-stranded monomeric hepatitis B virus (HBV) plasmid 4al or dimeric plasmid d4al, respectively. Plasmid DNAs were linearized with the restriction enzyme Scal prior to hybridization. Fragment 1 is complementary to parts of the HBV polymerase-/X sequences, and fragment 2 to parts of the core sequences. HBV DNA thick line; vector Bluescript KS-' thin line.